UNIVERSITY OF CALGARY

Intestinal Epithelial Barrier Dysfunction in Colitis

by

Andrew Christopher Skinn

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN

PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MEDICAL SCIENCE

CALGARY, ALBERTA

JUNE, 2005

UNIVERSITY OF
CALGARY

Abstract:

The intestinal epithelium acts as a defensive barrier lining the mucosal surface exposed to the external environment. Epithelial dysfunction may lead to numerous inflammatory conditions. Environmental factors have been implicated in initiating and exacerbating intestinal disease, particularly in genetically susceptible individuals. The focus of the present study was to further investigate the role of mediators and epithelial ion transport in inflammatory models to better understand the mechanisms leading to the onset of disease. Nitric oxide (NO) has been shown to affect secretory function during inflammation. Here, we studied the effects of NO on the trafficking of the chloride channel, cystic fibrosis transmembrane conductance regulator (CFTR), to the apical membrane. NO inhibited cAMP-dependent CFTR trafficking in intestinal epithelial cells and correlated temporally with changes in short circuit current in Ussing chamber studies. NO was also shown to selectively disrupt the tight junction protein ZO-1, but this did not lead to changes in epithelial permeability or bacterial translocation. Having assessed the effect of NO on epithelial function, we next explored barrier function in an animal model of intestinal bacterial infection. *Citrobacter rodentium* caused colitis and NO-independent epithelial dysfunction in mice. The epithelial secretory response to carbachol at 3 and 7 days post-infection was significantly reduced, but these changes were not dependent on inducible nitric oxide synthase (iNOS) or cyclooxygenase (COX)-2 expression. Since luminal pathogens and bacterial translocation have been implicated as possible causative factors in chronic inflammatory disorders, we next assessed

the mucosal barrier function of the epithelium in a murine model of radiation enteropathy together with *C. rodentium* infection. Bacterial translocation leading to systemic infection was significantly increased in infected animals exposed to ionizing radiation. However, these changes occurred without changes to colonic permeability. Taken together, these findings add significantly to our understanding of inflammation at both the cellular and whole animal levels with respect to mediators and functional properties of the intestinal epithelium.

Acknowledgments

This work was funded by operating grants from the Canadian Institutes of Health Research (CIHR), the Crohn's and Colitis Foundation of Canada and a Health Research Foundation/CIHR MD/PhD studentship.

I would like to thank my supervisor, Dr. Wallace MacNaughton for his guidance over the past few years and for making the lab a positive working environment. To everyone that has been in the lab, Kelly Cushing, Michelle Buresi, Kristen MacEachern, Hongying Wang, Jacques van der Merwe, Samara Freeman, and Michelle Seymour, thank you for your assistance, friendship and helping to make the lab an enjoyable place to work. My committee and the Mucosal Inflammation Research Group were always there for support and guidance and I am grateful for being part of such an outstanding group of people. I would also like to thank Dr. Philip Sherman for all of his assistance while on sabbatical in helping establish the *Citrobacter rodentium* model in Calgary and his ongoing advice, Laurie Cellars and Aundria Hood for assistance with the mouse procedures, and Stella Zamuner for her assistance with Western blot protocols.

I especially need to thank my mother, Cheryl, and my brother, Matt, for the support they have constantly provided and for encouraging me to keep going. I thank Amanda for always supporting my decisions, even when they involve moving across the country. The love and encouragement she has provided over the years cannot be measured. To the rest of my friends and family, thanks for always being there and helping me in more ways than you probably know.

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List of Symbols, Abbreviations and Nomenclature

AC	Adenylate cylcase
A/E	Attaching and Effacing
ATP	adenosine tri-phosphate
CaCC	Calcium-activated chloride channel
cAMP	Cyclic adenosine monophosphate
CCH	Carbachol
CD	Crohn's Disease
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony-forming units
cGMP	cyclic guanosine monophosphate
ClC	Chloride channel
COX	Cyclooxygenase
CR	*Citrobacter rodentium*
DNA	Deoxy-ribose nucleic acids
DSS	Dextran sodium sulfate
EFS	Electrical field stimulation
ENS	Enteric nervous system
EHEC	Enterohemorrhagic *Escherichia coli*
eNOS	Endothelial nitric oxide synthase
EPEC	Enteropathogenic *Escherichia coli*

Esp	*Escherichia coli* secreted protein
FSK	Forskolin
GI	Gastrointestinal
Gy	Gray
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
Isc	Short circuit current
JAM	Junctional adhesion molecule
LEE	Locus for enterocyte effacement
LPS	Lipopolysaccharide
MLCK	Myosin light chain kinase
MPO	Myeloperoxidase
mRNA	messenger RNA
NKCC	Sodium potassium 2 chloride co-transporter
nNOS	Neural nitric oxide synthase
NO	Nitric Oxide
NOC	Nocodazole
NOS	Nitric oxide synthase
OCTN	Organic cation transporter
PAR	Proteinase-activated receptor

PBS	Phosphate buffered saline
PD	Potential difference
PG	Prostaglandin
PKA	cAMP dependent protein kinase A
PKC	Protein kinase C
PLA_2	Phospholipase A_2
RNA	Ribonucleic acid
SCBN	Small intestinal cells of BN
TMCH	Transmissible murine colonic hyperplasia
TNBS	Trinitrobenzene sulfonic acid
TNF-α	Tumour necrosis factor-alpha
TTBS	Tween-Tris buffered saline
UC	Ulcerative Colitis
ZO	Zona occludens

Chapter 1: General Introduction:

1.1 Intestinal Inflammation – Genetics and Environment:

The gastrointestinal tract is a site of complex and divergent interactions. A large surface area is required to maximize nutrient absorption, but it is this same feature that contributes to the susceptibility of the gastrointestinal tract to pathogens and antigens. Thus, an efficient and effective host defense system is critical to prevent infection and the inflammation and tissue damage that occur as a result. Nevertheless, while infectious diseases of the gut are relatively under control in developed countries, idiopathic inflammatory conditions are on the rise (MacDonald and Monteleone, 2005). It is thought that both genetic and environmental factors play a role in the disruption of the balance between the commensal microflora and the mucosal immune system leading to the onset of these diseases.

1.1.1 Intestinal Inflammation:

The single layer of intestinal epithelial cells (IECs) that lines the gastrointestinal tract participates, along with dendritic cells and other components of the mucosal immune system, in differentiating between pathogens and commensal microflora. Under noninflammatory conditions, the epithelium is not only involved in normal barrier function, it also “samples” luminal contents in order to identify invading pathogens. Luminal antigens are constantly being presented to T lymphocytes to challenge the immune system even when an immune response is not required (Mumy and McCormick, 2005).

The innate immune system is responsible for recognition of pathogen-associated molecular patterns (PAMPs). This is achieved by pattern-recognition receptors on the surface of IECs (Medzhitov and Janeway, 2000). Toll-like receptors (TLRs) in humans resemble the Toll receptors of *Drosophila melanogaster* that play a role in defense against fungal and bacterial infection. There have been 10 identified mammalian TLRs that recognize PAMPs (Akira, 2003). TLR4 and TLR5 have generated the most interest in terms of intestinal inflammation. TLR4 recognizes lipopolysaccharide (LPS) in the outer cell membrane of Gram-negative bacteria (Takeuchi et al., 1999). TLR5 recognizes flagellin which is the major protein in bacterial flagella (Hayashi et al., 2001). Upon activation of TLRs, intracellular signaling events lead to activation of the transcription factor, NF-κB, resulting in the expression of many genes involved in the proinflammatory response (Faure et al., 2000). Nod receptors present in the cytosol can also signal and activate NF-κB and are thought to be involved in invasive pathogenic infections due to their intracellular location (Mumy and McCormick, 2005).

Following recognition of pathogens, IECs produce various signaling molecules to recruit and activate numerous immune cells leading to an inflammatory response. NF-kB activation results in the expression of proinflammatory cytokines and chemokines (Faure et al., 2000). These proteins, produced by IECs, act as chemoattractants for neutrophils, macrophages and dendritic cells. The most numerous of the leukocytes are neutrophils that produce proteases and reactive oxygen intermediates to destroy bacteria. IECs

secrete neutrophil chemoattractants, such as IL-8, to recruit neutrophils to the site of infection (Eckmann and Kagnoff, 2001; Zhang et al., 2003). Although the substances released by neutrophils are effective at killing bacteria, these substances can also damage the intestinal epithelium leading to intestinal inflammation during excessive neutrophil infiltration (Mumy and McCormick, 2005). IECs also produce the macrophage chemoattractants MCP-1 and MIP-1β leading to the recruitment of macrophages to the site of infection (Eckmann and Kagnoff, 2001). Macrophages engulf and destroy pathogens, but they also produce the proinflammatory cytokines IL-1 and TNF (Eckmann and Kagnoff, 2001).

Besides protein mediators of inflammation, IECs also produce eicosanoids in the host response to antigens. These lipid mediators include prostaglandins (PGs), leukotrienes (LTs) and lipoxins (LXs) (Mumy and McCormick, 2005). Upon release of arachidonic acid from the cell membrane, cyclooxygenases (COXs) and lipoxygenases (LOs) produce PGs and LTs, respectively. Following exposure to intestinal pathogens such as *Salmonella sp.*, PGH synthase-2 and COX-2 are upregulated producing increased PGE_2. PGE_2 then upregulates chloride secretion resulting in increased water movement leading to diarrhea (Berkes et al., 2003). Eicosanoids can also be anti-inflammatory in function. For example, LXA_4 can downregulate NF-κB-mediated IL-8 production suggesting that it may play a role in downregulating intestinal inflammation and perhaps prevent extensive damage to the host tissue (Kucharzik et al., 2003).

1.1.2 Inflammatory Bowel Disease:

Inflammatory bowel disease (IBD) in humans can be divided into two main types: Crohn's disease (CD) and ulcerative colitis (UC) (Podolsky, 2002). While clinical symptoms of the two diseases include diarrhea, cramping and abdominal pain, there are important differences in disease etiology. Crohn's disease occurs in patches of transmural inflammation that can occur anywhere from the mouth to the anus and are often interrupted by areas of normal healthy tissue (Karlinger et al., 2000). The inflammation characterizing ulcerative colitis begins at the rectum and extends proximally in a continuous fashion affecting only the colonic mucosa. In most cases, patients experience recurrent flare-ups and remissions of the disease. These diseases do not have a known cure or cause, although recent studies have looked at genetic factors associated with IBD, and CD in particular.

1.1.3 Genetic Susceptibility and IBD:

Crohn's disease susceptibility was shown to be mapped to a number of loci on several chromosomes (Wild and Rioux, 2004). In 2001, two studies showed that the locus on chromosome 16 is the Nod2 gene (Ogura et al., 2001; Hugot et al., 2001). In approximately 15% of CD patients, three polymorphisms of Nod2 are present. There is a 20- to 40-fold increase in the risk of developing CD for patient's carrying 2 copies of these alleles (Inoue et al., 2002). Nod2 is expressed in the cytosol of intestinal epithelial cells, macrophages and dendritic cells (Inohara and Nunez, 2003). The mechanism of action for Nod2 leads to

activation of NF-κB and it is thought that the mutations in Nod2 may lead to a decreased ability to kill bacteria in the gut (Hisamatsu et al., 2003). Therefore, it is tempting to speculate that in individuals with Nod2 mutations, this decreased ability to kill bacteria may lead to the triggering of immune mediators and thus initiate inflammation contributing to IBD. Another gene that has also been associated with CD is the gene encoding the organic cation transporter (OCTN) (Peltekova et al., 2004). Mutations in these genes lead to reductions in amino acids being transported across cell membranes. A third gene encodes the guanylate kinase DLG5 and its mutation is thought to have an effect on cell polarity (Stoll et al., 2004). Therefore, mutations in OCTN and DLG5 may be important in epithelial cell permeability and affect exposure of the mucosal immune system to bacterial products leading to progression of CD.

Although there is a statistical correlation between CD and risk-associated Nod2 alleles, only 8 to 17% of CD patients carry two copies of the alleles compared to 1% of the general population. Also interesting is that CD in Japan has been shown not to be associated with Nod2 (Inoue et al., 2002). These findings highlight the complexities of CD and indicate that other factors such as environmental influences may also play a role in the progression of this disease.

1.1.4 Environmental Factors and IBD:

In CD patients, increased bacterial translocation into deeper layers of the mucosa has been demonstrated and may be of pathophysiological relevance (Boudeau et al., 2003). The evidence that environmental factors such as bacteria play a major role in the progression of intestinal inflammation is further

strengthened by studies showing that inflammation does not develop in germ-free animal models (Guarner and Malagelada, 2003). The two ideas of “genes and germs” are converging since it is becoming more evident that a luminal pathogen is likely affecting genetically susceptible individuals leading to onset of disease.

The question can then be raised as to whether IBD results from a loss of epithelial barrier function or if barrier dysfunction is a secondary defect due to intestinal inflammation. In IL-10 deficient mice, epithelial permeability is increased before signs of overt inflammation appear (Madsen et al., 1999). In addition, CD patients show increased gut permeability, as do first degree relatives and spouses of these patients (May et al., 1993; Breslin et al., 2001; Soderholm et al., 1999), although, it is still not known if asymptomatic individuals with high permeability will necessarily develop IBD in the future. It is also possible that the barrier defect is present due to undetectable low grade inflammation or that other immune mediators may be increasing epithelial permeability (McKay and Baird, 1999).

A consequence of increased intestinal permeability is an increased exposure to pathogens in the lumen. While many pathogens, such as *Myobacterium paratuberculosis*, have been proposed as the cause for IBD, a single pathogen has yet to be identified as being solely responsible for CD or UC. It is now thought that the “pathogen” responsible for IBD may be one’s own commensal microflora. Again, some of the key evidence for this hypothesis is that animals maintained in germ-free conditions demonstrate decreased disease

severity in models of intestinal inflammation (Bouma and Strober, 2003). Some CD patients have also been shown to initiate an immune response against their own flora suggesting that tolerance to commensal bacteria may be lost during intestinal inflammation (Duchmann et al., 1995). Stressful life events are coincident with relapses of active IBD (Collins, 2001), and recent studies have demonstrated that intestinal epithelial cells undergoing metabolic stress show increased IL-8 release and increased epithelial permeability to the commensal non-pathogenic *E. coli* HB101 strain (Nazli et al., 2004).

Another "environmental" factor that leads to intestinal inflammation is exposure to ionizing radiation. Acute radiation enteritis is a problem in some cancer patients since radiotherapy is still a mainstay of treatment of cancers in the abdominopelvic region. Intestinal exposure to ionizing radiation has been shown to cause bacterial translocation (Guzman-Stein et al., 1989), although the mechanism by which the bacteria cross from the lumen into the lamina propria is not known. Diarrhea is also a consequence of irradiation and may be a result of numerous factors including bacterial overgrowth (Bismar and Sinicrope, 2002). Many patients developing acute enteritis following radiotherapy will progress to chronic complications. These include fistulae and stricture formation as well as bowel obstruction (Novak et al., 1979; Coia et al., 1995). These symptoms obviously adversely affect quality of life for the patient and often require surgical intervention. Bacterial colonization and translocation may play a role in this progression.

It is clear that intestinal inflammation, in both IBD and radiation enteropathy, is a complex situation likely involving a breakdown in host defense leading to increased exposure of the lamina propria to a luminal factor. Clinically, both IBD patients and individuals undergoing radiotherapy show increases in bacterial translocation. Therefore, if we better understand the mechanisms involved in epithelial dysfunction, we can develop strategies to prevent bacterial translocation and the subsequent development of disease.

1.2 The Role of the Epithelium as a Defensive Barrier – An Overview:

The human gut has numerous features to prevent luminal antigens from entering into the tissues. Upon surviving the acidic environment of the gut and penetrating the semipermeable mucous layer, pathogens encounter the intestinal epithelium. The epithelium acts as a defensive barrier lining the mucosal surfaces of organs exposed to the external environment, such as the gastrointestinal tract and airways. This host defense function of the epithelium is achieved in several ways. First, the epithelial layer has a low level of permeability due to intercellular junctions (tight junctions) between the cells (Anderson and Van Itallie, 1995). In the gastrointestinal tract, these junctions prevent harmful materials, such as antigens and toxins, which are present in the lumen, from translocating into the intestinal mucosa. Second, secretory immunoglobulin-A (IgA) targets antigens at the mucosal surface, and is the main humoral component of the mucosal immune system. The IgA response is important in preventing bacteria from translocating into the mesenteric lymph

nodes (Fagarasan and Honjo, 2004). Third, secretion of ions, such as chloride, from crypt cells creates osmotic pressure allowing fluid to move into the lumen. This fluid movement prevents bacteria from colonizing and antigens from crossing into the lamina propria (Greger, 2000). Constant regulation of water absorption and secretion is important to meet cellular conditions and requirements. For example, bacterial infection leads to an increase in chloride and water secretion as a mechanism to dilute and flush away the bacteria (Wood, 1993). This is accomplished by numerous secretagogues acting to either up- or down-regulate ion secretion (Figure 1.1).

Secretory dysfunction or impaired absorption may lead to disruption of barrier function. Hyperresponsiveness to secretagogues can occur leading to excess secretion. This is the case with diarrhea due to cholera infection leading to excess ion and water loss (Speelman et al., 1986). On the other hand, hyporesponsiveness to secretagogues impairs chloride and water secretion. This is observed in inflammatory bowel disease where decreased response to secretagogues is associated with an increased incidence of bacterial translocation into the lamina propria (Kunzelmann and Mall, 2002).

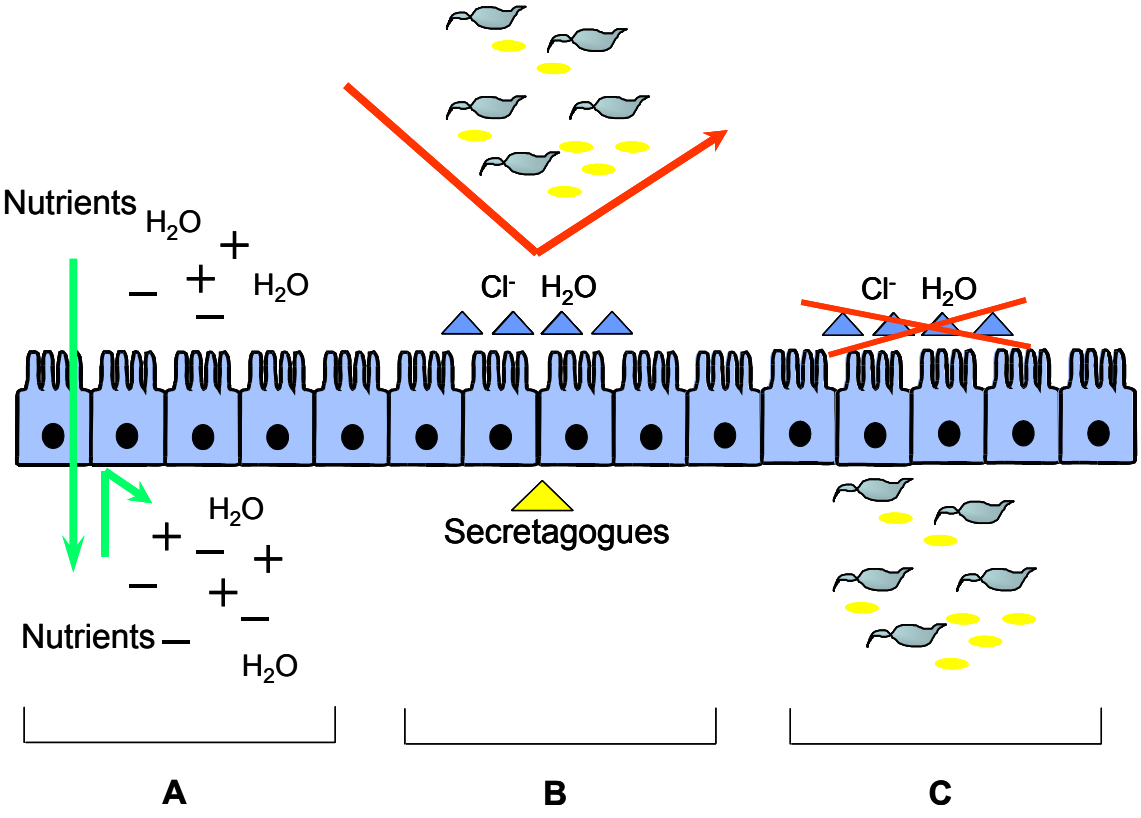


Figure 1.1. The Epithelium as a Defensive Barrier. The epithelium lines mucosal surfaces, such as the gastrointestinal tract and acts as the first line of protection to the external environment. Tight junctions exist between the cells to help prevent pathogens from crossing the epithelium. A. The epithelium regulates the absorption of water, nutrients and ions. B. Secretion of ions creates osmotic pressure allowing fluid to move into the lumen. Secretagogues enhance this action to flush out pathogens. C. Hyporesponsiveness to secretagogues prevents fluid from moving across the epithelium which can lead to bacteria and other pathogens crossing into the lamina propria.

1.3 Tight Junctions:

Tight junctions (TJs) are intercellular complexes that function as a barrier to restrict movement of material between epithelial cells. The complex consists of membrane-spanning proteins as well as intracellular proteins. The intracellular complex is composed of many different proteins. These include the zonula occludens proteins (ZO-1, ZO-2, and ZO-3), cingulin, 7H6, sympleken and ZA-1TJ as well as many signaling molecules. The membrane-spanning molecules are occludin, members of the claudin family, junctional adhesion molecule (JAM) and adenovirus receptor (CAR). Figure 1.2 is a schematic structure of the TJ. Disruption of this complex can lead to increased epithelial permeability (Mitic et al., 2000).

1.3.1 Tight Junction Integral Membrane Proteins:

Occludin forms two extracellular loops and a short cytosolic loop as it spans the cellular membrane. Phosphorylated occludin is expressed in the TJ and nonphosphorylated occludin shows punctate immunofluorescent staining in the basolateral membrane (Sakakibara et al., 1997). It is believed that PKC is involved in the phosphorylation of occludin since exposure to PKC agonists results in TJ assembly and occludin phosphorylation, whereas this process is inhibited by PKC inhibitors (Andreeva et al., 2001). Overexpresseion of occludin in epithelial cells results in increased transepithelial resistance (TER) (Balda et al., 1996; McCarthy et al., 1996). However, occludin-deficient mice do not show structural or functional TJ abnormalities (Saitou et al., 2000) which suggests that

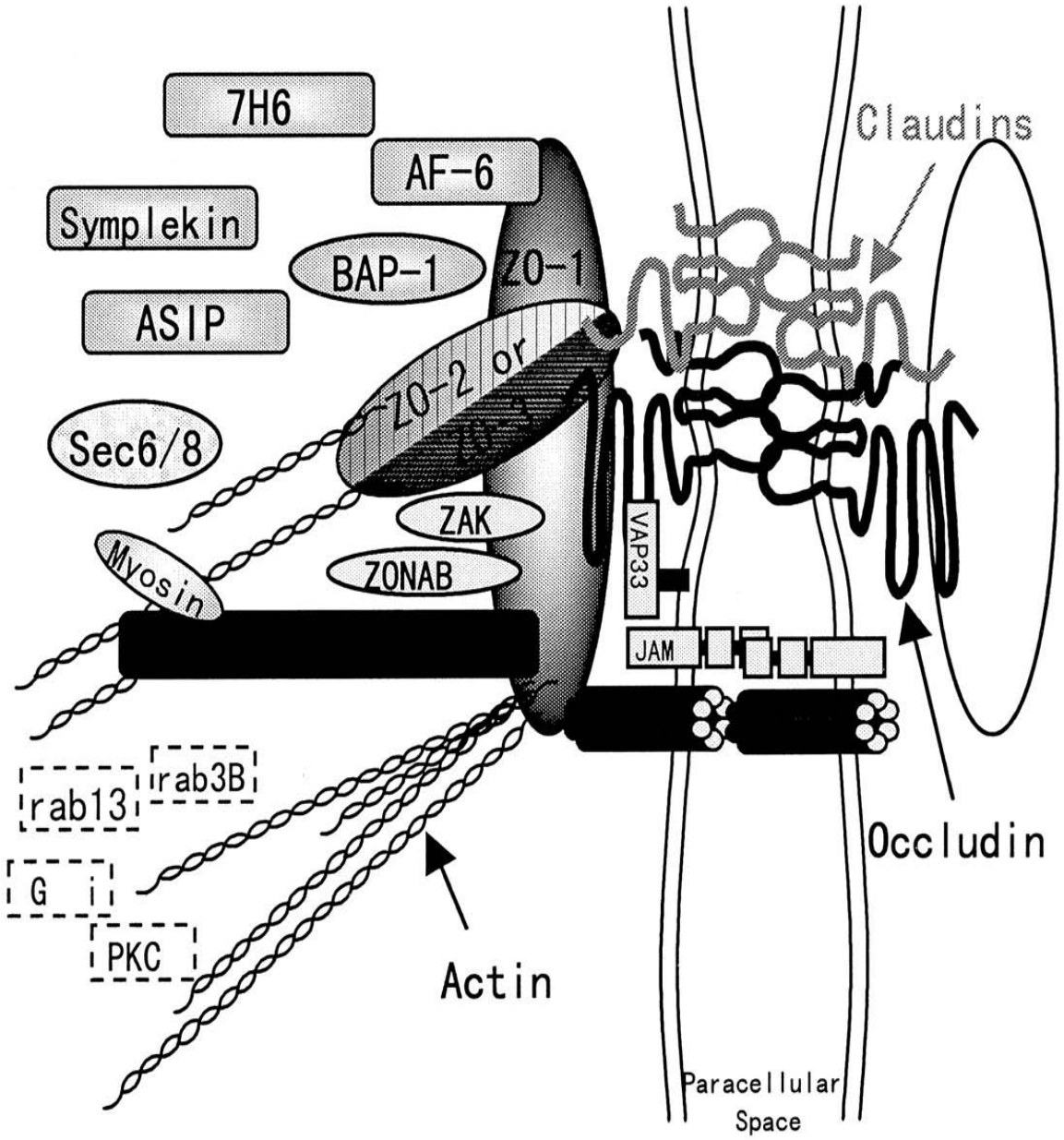


Figure 1.2. Structure of the tight junction. Tight junctions are intercellular complexes that function as a barrier to prevent movement of material between the cells of the epithelial layer (Mitic et al., 2000).

other proteins can maintain TJ integrity. This observation led researchers to discover the claudin family of TJ proteins (Furuse et al., 1998).

Claudins, like occludin, span the cellular membrane with two extracellular loops and a short cytoplasmic loop. The carboxy terminus of all claudins binds to PDZ domains of proteins including those of the zonula occludens (Itoh et al., 1999). Claudin-1, for example, interacts with ZO-1 and this interaction is necessary for proper incorporation of ZO-1 into the TJ (McCarthy et al., 2000). Genomic cloning has shown that there may be up to 24 members of the claudin family (Tsukita et al., 2001) and differential expression of these isoforms may play a role in ion selectivity and barrier function in different cell types. For example, claudin-4 expressed in MDCK cells resulted in increased TER correlating with a decrease in sodium, but not chloride permeability (Van Itallie et al., 2001). Due to the varying features of the various claudin proteins, it is important to investigate various claudins when looking at changes in epithelial permeability due to tight junction protein expression.

While occludin and the claudins are the main structural integral membrane proteins of the TJ, other proteins are also present. JAM proteins have been shown to reside in the cellular membrane at the tight junction with a single transmembrane domain, two extracellular Ig domains, and an intracellular domain with a PDZ binding motif. It is thought that JAM proteins contribute to the organizational process of TJ formation (Schneeberger and Lynch, 2004). CAR also has a single transmembrane domain and its function in the TJ is still

unknown although it is known to be a target of pathogens such as viruses and bacteria (Schneeberger and Lynch, 2004).

1.3.2 ZO Proteins:

ZO-1 was the first TJ-associated protein to be identified and is the most studied (Stevenson et al., 1986). ZO-1 binds with many other proteins including the claudins (Itoh et al., 1999) and ZO-2 and ZO-3 (Giepmans and Moolenaar, 1998). ZO-1 also binds to occludin providing a link between occludin and the actin cytoskeleton (Fanning et al., 1998). Due to the interactions of ZO-1 with many other TJ-associated proteins (see Figure 1.2), it is clear that ZO-1 has a function in protein scaffolding but its precise function remains to be investigated. ZO-2 and ZO-3 are thought to be involved in further intracellular signaling pathways, but these pathways have not been fully elucidated (Schneeberger and Lynch, 2004).

1.3.3 Regulation of Tight Junctions:

Accumulating evidence suggests that myosin light chain kinase (MLCK) is a common intermediate in numerous pathways altering paracellular permeability. Increased paracellular permeability occurs in response to Na^+-glucose co-transport by the Na^+-glucose cotransporter, SGLT1 (Berglund et al., 2001). This process is driven by MLCK-mediated phosphorylation of myosin II regulatory light chain. The cytokines interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) are upregulated in inflammatory bowel disease and lead to rearrangement of tight junction proteins including ZO-1, occludin, claudin-1 and claudin-4

(Clayburgh et al., 2004). The resulting change in paracellular permeability is reversible by a myosin light chain kinase inhibitor, demonstrating another pathway leading to the MLCK intermediate (Zolotarevsky et al., 2002).

1.4 Mechanisms of Chloride Secretion:

Transport of fluid in the gastrointestinal tract is achieved through osmotic gradients created by the movement of electrolytes and other solutes. Chloride secretion is considered the predominant process involved in luminally directed fluid movement and sodium is the predominant ion in fluid absorption (Barrett and Keely, 2000).

An inwardly directed sodium gradient is created by the Na^+/K^+-ATPase located in the basolateral membrane. This sodium gradient allows chloride to move into cells across the basolateral membrane by the sodium, potassium, 2-chloride-cotransporter, NKCC1. The result is accumulation of chloride within the cell, establishing a concentration gradient favouring movement of chloride out of the cell when apical chloride channels are activated. Potassium channels present in the basolateral membrane allow for recycling of potassium and prevent the cell from depolarizing when chloride exits the cell apically (Barrett and Keely, 2000). A simplified illustration of this system is shown in Figure 1.3.

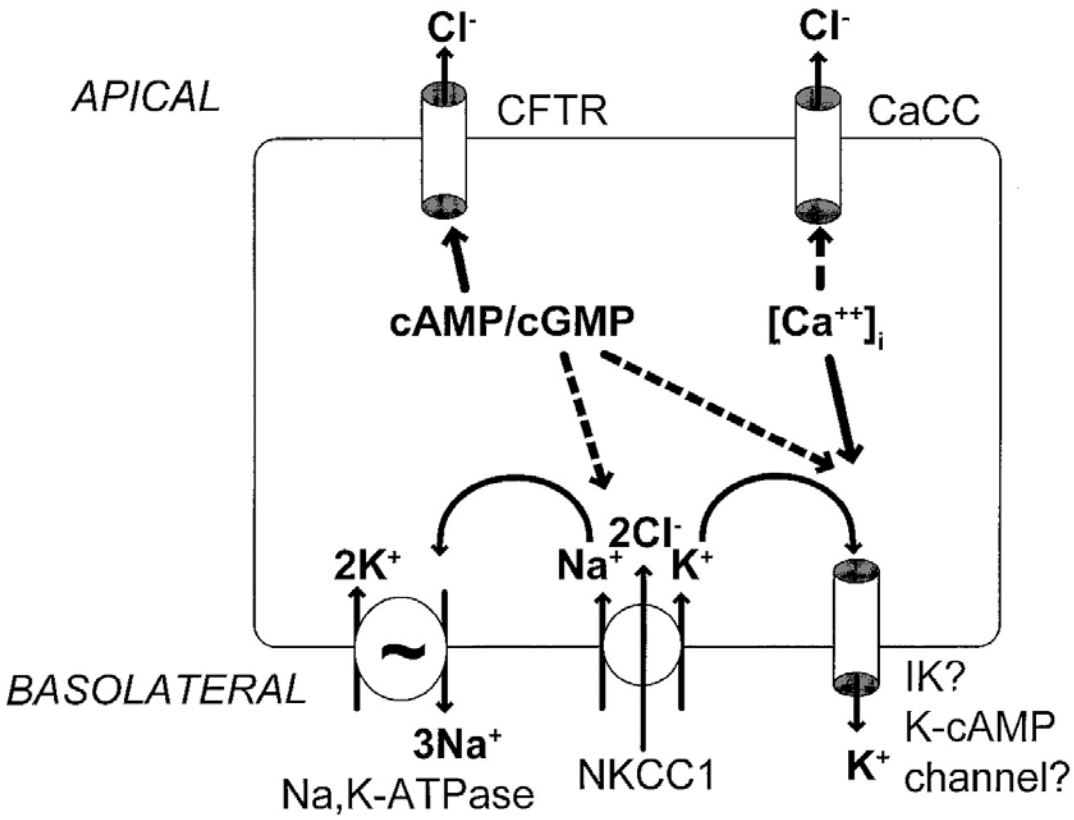


Figure 1.3. Mechanisms of chloride secretion in intestinal epithelial cells. Chloride secretion is driven by the Na^+K^+ATPase. Chloride is transported into the cell by NKCC1 while maintaining intracellular Na^+ concentrations. K^+ is transported out of the cell at the basolateral membrane by K^+ channels. Upon accumulation within the cell, chloride is transported from the cell at the apical membrane via CFTR and CaCl channels. This process is regulated by intracellular cAMP, cGMP, and calcium. (Figure from Barrett and Keely, 2000)

The amount of chloride secretion taking place at a given time is dependent on intracellular ion concentrations as well as channel activation. For example, calcium-dependent chloride secretion involves calcium acting to open apical calcium-dependent chloride channels (CaCC) and basolateral calcium-dependent potassium channels (Gruber et al., 1998). The intracellular signaling pathway for calcium-dependent chloride secretion is through phospholipase C (PLC) and inositol triphosphate (IP_3). For other chloride channels such as the cystic fibrosis transmembrane conductance regulator, CFTR, chloride secretion may be regulated by covalent modification (Berger et al., 1993; Berger et al., 1991). Other mechanisms by which chloride secretion through CFTR is regulated will be discussed below.

1.5 Chloride Channel Regulation in Intestinal Secretion:

1.5.1 Cystic Fibrosis Transmembrane Conductance Regulator (CFTR):

Regulation of chloride secretion through CFTR is thought to occur through three processes: phosphorylation leading to channel opening, trafficking of CFTR to the membrane, and CFTR endocytosis. All processes occur predominantly via cyclic AMP (cAMP)-dependent mechanisms. There is also evidence suggesting a role for calcium in CFTR activation (Wagner et al., 1991; Berger et al., 1993). In these studies, fibroblasts stably transfected with CFTR were shown to be activated by both calcium-dependent and calcium-independent isoforms of protein kinase C (PKC). PKC was also shown to phosphorylate CFTR in vitro in these studies.

CFTR gating is controlled by intracellular concentrations of cAMP and by protein kinase A (PKA) activity (Picciotto et al., 1992; Bradbury, 2001). Phosphorylation of the R-domain of CFTR by PKA results in nucleotide binding and hydrolysis (Figure 1.4). Following both of these steps, the channel opens and chloride moves out of the cell. It has also been suggested that other protein kinases such as PKC may also play a role in increasing CFTR activation by PKA (Fischer and Machen, 1996).

Trafficking of transport proteins to the apical membranes of cells plays an important role in upregulation of ion secretion. This is the case for cystic fibrosis (CF) where mutations in the CFTR gene lead to the development of disease (Riordan et al., 1989). Although many mutations in this gene are possible, the ΔF508 mutation is homozygous in approximately 70% of individuals with CF and is the mutation that occurs in approximately 90% of mutant CFTR alleles (Mickle and Cutting, 1998). With this mutation, trafficking of CFTR to the apical plasma membrane is very inefficient (Puchelle et al., 1992) although the chloride channel component of CFTR remains partially functional (Li et al., 1993; Pasyk and Foskett, 1995). Also, even when the ΔF508-CFTR does insert in the membrane, it remains in the membrane for a much shorter time than does wild-type CFTR (Lukacs et al., 1993). Increasing CFTR expression at the plasma membrane has been studied experimentally due to the implications for the treatment of CF. Factors such as decreased temperature (Denning et al., 1992), butyrate compounds (Rubenstein et al., 1997), and DMSO and glycerol (Sato et al., 1996) have been shown to increase ΔF508-CFTR expression at the membrane.

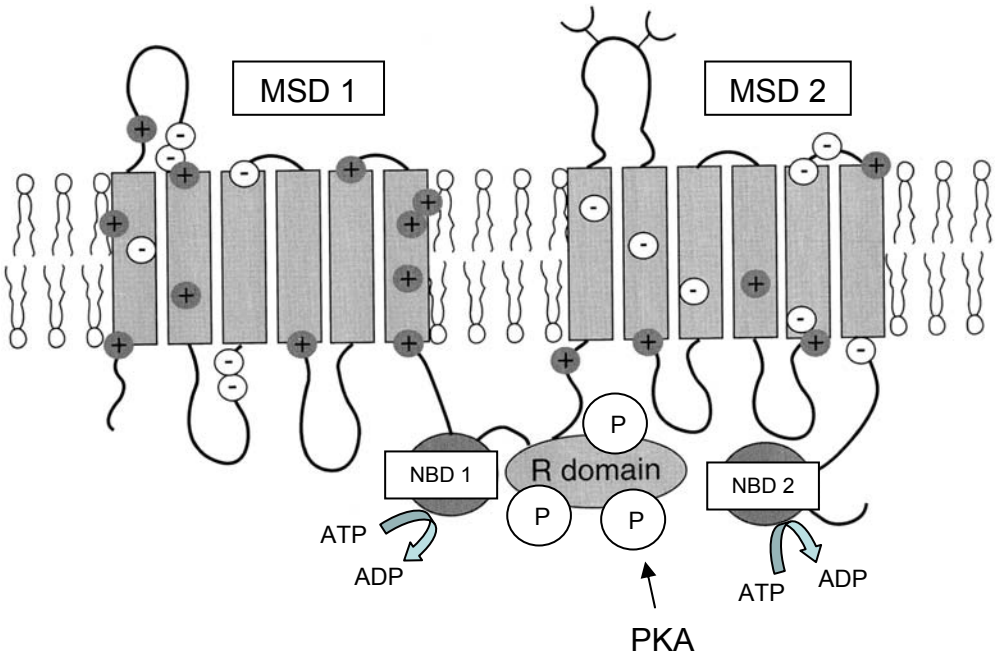


Figure 1.4. Structure of the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR has two membrane spanning domains, MSD 1 and MSD 2 and the channel opening in the middle. Upon phosphorylation of the R-domain by PKA, nucleotide binding and hydrolysis occur at the nucleotide binding domains, NBD 1 and NBD 2. This results in channel opening allowing chloride to move out of the cell. (Figure adapted from Dawson et al., 1999)

Trafficking of wild-type CFTR to the apical membrane, when studied experimentally in epithelial cell lines, appears to be dependent on cAMP. T84 cells stimulated with the adenylate cyclase activator forskolin exhibit increased CFTR immunoreactivity at the apical surface (Tousson et al., 1996). However, in other cell lines, such as non-polarized HT-29, (Morris et al., 1994) cAMP-dependent CFTR translocation is not observed. Besides polarity of the cell lines, some other variables are thought to be involved in the trafficking and processing of CFTR. For example, in experiments using *Xenopus* oocytes expressing the ΔF508 mutated form of CFTR, reduction in temperature restores processing of CFTR and trafficking of functional cAMP-regulated CFTR to the apical membrane is observed (Denning et al., 1992). The time point being investigated for CFTR trafficking is also important as it has been shown that recruitment of CFTR to the apical membrane in T84 cells can occur as rapidly as 30 seconds after exposure to the adenylate cyclase activator forskolin (Tousson et al., 1996).

Chloride secretion may also be regulated by endocytosis of CFTR which is inhibited in the presence of cAMP (Bradbury et al., 1992). Endocytosis of CFTR occurs in clathrin-coated vesicles following PKA and PKC phosphorylation (Prince et al., 1994). Degradation of CFTR takes place at the endoplasmic reticulum in proteasomes (Jensen et al., 1995). These processes, translocation to the apical membrane, endocytosis and degradation will all determine the amount of CFTR which will be present at the apical membrane, and which could be activated for chloride secretion.

1.5.2 Calcium-activated Chloride Channels (CaCCs):

Although CFTR is the predominant channel in the intestine regulating chloride transport and thus water movement, there are other chloride channels that contribute to the overall ion flux. Calcium-activated chloride channels (CaCCs) are also present at the apical surface and are activated by increases in the concentration of calcium in the cytosol.

The exact mechanism of CaCC activation is still being elucidated. Early studies have shown that increases in intracellular calcium activate basolateral potassium channels leading to increased NKCC1 activity and chloride movement by CFTR channels still present and open at the apical membrane (Anderson et al., 1992). In other experiments, however, a chloride secretory response to increases in calcium has been demonstrated in a mouse model of cystic fibrosis where functional CFTR channels are not available (Grubb and Gabriel, 1997; Rozmahel et al., 1996). Gene cloning has also identified multiple isoforms of CaCC with at least one of them being expressed in the human intestine (Gruber 1998) and activated by increases in cytosolic calcium.

Studies have shown that calcium can both stimulate and inhibit chloride secretion by different mechanisms. In the case of stimulation of chloride secretion, agonists that evoke increases in intracellular calcium, such as carbachol binding to an M_3-muscarinic receptor, cause activation of PLC leading to production of IP_3 and diacylglycerol (Nishizuka, 1984). IP_3 stimulates release of calcium from intracellular stores, which activates calcium-calmodulin-dependent protein kinase (CaMK) which in turn activates CaCC (Arreola et al.,

1998). However, increases in intracellular calcium will transactivate the epidermal growth factor receptor (EGFr). Activation of EGFr leads to recruitment of ERK1/2 isoforms of MAPK which can decrease the chloride secretory response (Keely et al., 2000). It should be noted, however, that this response to EGFr activation is cell-line dependent. Previous work in our lab in a different cell line demonstrated that EGFr transactivation and ERK1/2 activation leads to increased chloride secretion (Buresi et al., 2001).

1.5.3 Chloride Channel (ClC)-2:

While the function and regulation of many of the chloride channels (ClC) remains to be understood, ClC-2 is one member of this channel family that has been studied in some detail. This channel is expressed in most cells that express CFTR and has generated interest in the cystic fibrosis (CF) field as a possible mechanism for cells to overcome chloride secretion abnormalities associated with CF (Wills and Fong, 2001). ClC-2 has been found at the apical membrane and shown to be capable of vectorial movement of chloride (Blaisdell et al., 2000). Recent studies have shown that IFN-γ can regulate ClC-2 in lung epithelial cells by stabilizing ClC-2 mRNA transcripts (Chu et al., 2004).

ClC-2 is activated upon cell swelling and hyperpolarizing voltages. Phosphorylation sites exist on the channel for activation by PKA (Cid et al., 1995; Sherry et al., 1997) although evidence for this activation has not been conclusive (Tewari et al., 2000). It does appear though that ClC-2 is capable of moving chloride across the apical membrane, although its contribution to overall chloride secretion, when CFTR is fully functional, is still unknown.

In healthy cells, regulation of chloride secretion, predominantly by CFTR, can be accounted for by the cAMP-dependent processes discussed above. However, in situations of inflammatory disease, it is likely that other factors and mediators will also regulate chloride secretion. Of interest is the role that some inflammatory mediators have in rendering the epithelium hyporesponsive to secretagogues. This has important implications in terms of the role of the epithelium as a barrier to bacterial translocation precipitating or exacerbating inflammation. Nitric oxide is one such mediator and its role in mediating chloride secretion in conditions of inflammation will be discussed in the following section.

1.6 Nitric Oxide and Nitric Oxide Synthases:

Nitric oxide (NO) is a gas that readily crosses cell membranes. NO has an unpaired electron in its outer shell making it a highly reactive oxyradical. Cleavage of a terminal guanadino group on *L*-arginine by the oxidative enzyme nitric oxide synthase (NOS) produces NO and *L*-citrulline (Figure 1.5) (Beckman et al., 1990; Forstermann et al., 1994). There are three isoforms of NOS.

Neural/brain NOS (nNOS, bNOS or NOS I) is a constitutively expressed calcium / calmodulin-dependent NOS producing low (nM) concentrations of NO. nNOS is found in the brain and neurons that possess NMDA receptors. Once these receptors are stimulated, calcium influx occurs, NO synthesis is triggered and guanylate cyclase is activated in Purkinje cells. nNOS is also present in the enteric nervous system where it acts as a nonadrenergic, noncholinergic neurotransmitter. Nerve stimulation in the myenteric plexus leads to NO

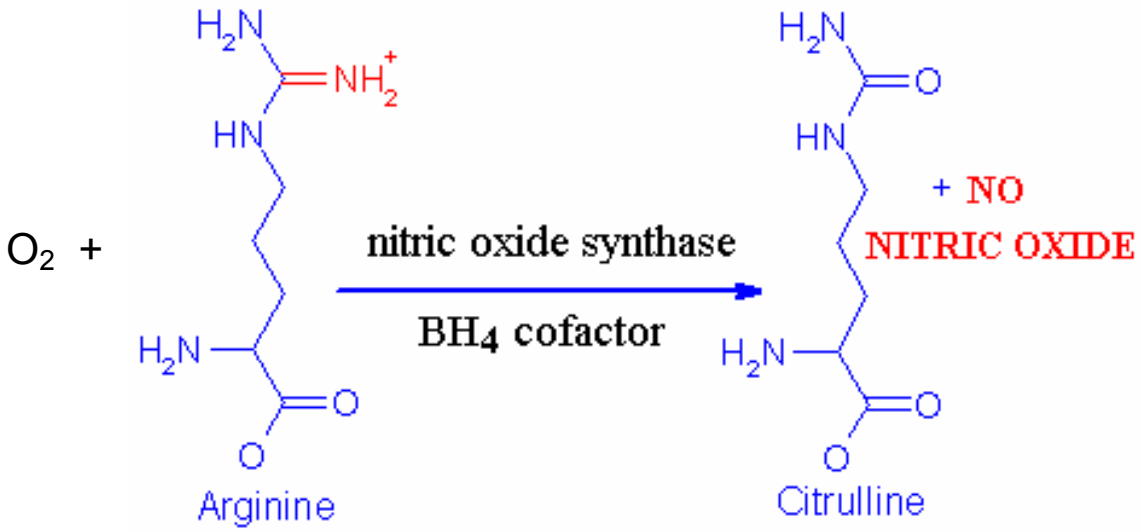


Figure 1.5. Enzymatic Reaction of Nitric Oxide Synthases. Cleavage of a terminal guanadino group on *L*-arginine by the oxidative enzyme nitric oxide synthase (NOS) produces NO and *L*-citrulline. There are three different NOS isozymes.

production by nNOS and plays an integral role in gastrointestinal function. For instance, reduction of nNOS expression and subsequent impaired local production of NO can lead to motility disorders in the GI tract (Takahashi, 2003). In this way, it is thought that the nanomolar concentrations that are generated by the enteric nervous system act to protect the gastrointestinal tract and prevent onset of disorders. In the mouse model of dextran sulphate sodium (DSS) colitis, nNOS-deficient animals showed more severe disease and mortality than wild-type animals (Beck et al., 2004).

Endothelial NOS (eNOS or NOS III) is a constitutively expressed calcium / calmodulin-dependent NOS producing low (nM) concentrations of NO. eNOS is expressed in many different cells including endothelial cells, epithelial cells, smooth muscle cells and neurons. The effects of NO produced by eNOS are variable. Regulation of vascular tone is the main function of eNOS-derived NO, although it has also been shown to regulate leukocyte adhesion, smooth muscle cell proliferation and platelet aggregation (Albrecht et al., 2003). eNOS-deficient mice were shown to be protected from the inflammatory response seen in the mouse model of DSS colitis (Beck et al., 2004).

Inducible NOS (iNOS or NOS II) is a calcium-independent NOS induced by lipopolysaccharide (LPS) and inflammatory cytokines producing high (μM) concentrations of NO. iNOS is expressed in many different cells including epithelial cells, endothelial cells, macrophages and neurons. In inflammatory conditions in the lung (Belvisi et al., 1995) and colon (MacNaughton et al., 1998; Guihot et al., 2000), iNOS activity levels have been shown to be elevated. iNOS-

deficient mice are protected from the inflammatory response in the mouse model of DSS colitis (Beck et al., 2004). The function of iNOS and iNOS-derived NO in inflammatory conditions will be discussed in more detail below.

As demonstrated by the many different functions of NO derived from the different nitric oxide synthases, NO is an important molecule. However, its production is not always seen as being beneficial. eNOS and nNOS are considered constitutive nitric oxide synthases and they produce NO in nanomolar concentrations. Although these NOSs are constitutively expressed, their basal levels of NO production can be regulated by temperature, ischemia and inflammation (Albrecht et al., 2003). iNOS on the other hand is considered the inducible nitric oxide synthase. Although some cells such as the epithelium of the lung constitutively express iNOS, it is expressed and produces its highest levels of NO (μM) in inflammatory cells after induction by inflammatory mediators such as cytokines. Therefore, in general, the constitutive nitric oxide synthases producing nanomolar amounts of NO are considered to have a role in normal function and protection whereas iNOS produces micromolar amounts of NO that can play an important role in host defense, but can also have damaging effects (Schirgi-Degen and Beubler, 1998).

1.7 The Role of Nitric Oxide in the Regulation of Chloride Secretion:

Secretion of ions, such as chloride, by the epithelium creates an osmotic gradient which acts to flush out invading pathogens. The role of nitric oxide in epithelial secretory function has been studied in detail recently. Many of these studies have focused on intestinal ion transport in models of inflammation since

the expression of iNOS is known to be triggered by inflammatory cytokines. Also, it has been demonstrated that disruption of the secretory function of the epithelium impairs its ability to act as a first-line host defense. As a result, increased bacterial translocation is observed in these models. If NO is implicated in secretory dysfunction, the impact could clearly be a role in bacterial overgrowth and the subsequent translocation. Studies on the role of NO in epithelial transport have been conflicting. Some evidence suggests that NO stimulates chloride secretion whereas other studies suggest it inhibits chloride secretion.

Studies looking at segments of guinea pig intestine mounted in Ussing chambers showed that serosal exposure to NO donating compounds resulted in chloride-dependent increases in short circuit current (MacNaughton, 1993). This was confirmed in another study using rat colon (Wilson et al., 1993). When the neural blocker tetrodotoxin was used as a pretreatment in the guinea pig study, no significant difference was observed upon addition of the NO donating compound suggesting that in guinea pigs, NO was exerting its effect independently of enteric nerves. However, in another study using rat distal colon, the same increase in short circuit current was observed to NO donors, however, tetrodotoxin did inhibit this response (Tamai and Gaginella, 1993). This suggests that the response to NO in the intestine may have different mechanisms of action depending on the species. In a later study using human colonic tissue, NO donors again stimulated a chloride-dependent increase in short circuit current (Stack et al., 1996). These studies implicated enteric nerves

and prostanoid synthesis as a partial mechanism for the increase in chloride secretion.

Contrary to these observations, other studies have shown that nitric oxide has an absorptive effect. Many of these studies looked at the intestine in its basal state. In perfusion studies of rabbit ileum, inhibiting NO synthase activity caused water and ion secretion and was reversed upon addition of the nitric oxide synthase substrate L-arginine. When an NO donor was added, absorption of water and ions was observed (Barry et al., 1994). Similar results were observed in dog ileum (Maher et al., 1995). In a rat ligated jejunal loop model, the NOS inhibitor L-NAME induced net secretion of fluid in a dose-dependent manner (Schirgi-Degen and Beubler, 1995). In these studies, intra-arterial infusion of prostaglandin-E_2 (PGE_2) induced net secretion of fluid that was significantly enhanced by L-NAME. The secretion induced by PGE_2 was reversed by L-arginine and by the NO donor, sodium nitroprusside. Similar findings were shown following intraluminal infusion of cholera toxin in that the net fluid secretion was enhanced by L-NAME and blocked by L-arginine and sodium nitroprusside (Beubler and Schirgi-Degen, 1997). Therefore, it appears that at endogenous levels, NO may have an absorptive role.

Several studies have investigated the role of iNOS-derived NO in epithelial dysfunction in inflammation. In a mouse model of acute trinitrobenzenesulfonic acid (TNBS) colitis, expression of iNOS mRNA was significantly increased in the inflamed colons compared to healthy control mice (McCafferty et al., 1999). In the inflamed tissue, an increase in iNOS activity was also observed. Inflamed

colon compared to healthy tissue mounted in Ussing chambers showed a decrease in the change in short circuit current in response to electrical field stimulation, which depolarizes secretomotor neurons in the preparation to cause chloride secretion (MacNaughton et al., 1998). The selective iNOS inhibitor L-NIL partially reversed this decreased response suggesting that the secretory dysfunction in colitic animals as a result of increased iNOS activity is neurally mediated.

In a model of acute radiation enteropathy, colons from irradiated wild-type mice, but not from irradiated iNOS-deficient mice, showed a reduction in response to both electrical field stimulation and forskolin when mounted in Ussing chambers 3 days post-irradiation (Freeman and MacNaughton, 2000). iNOS mRNA expression and nitrate/nitrite concentrations were elevated in colons of irradiated mice. iNOS-selective inhibitors reversed this reduction in the wild-type mice. However, in this model, the colons did not exhibit an inflammatory response as measured by histology and myeloperoxidase activity. Therefore, NO is able to invoke secretory dysfunction even without the onset of an inflammatory response (Freeman and MacNaughton, 2000).

The cellular target of iNOS-derived NO following exposure to ionizing radiation injury has recently been elucidated. Two isoforms of adenylate cyclase (AC5, AC6) are selectively inhibited by NO and are expressed in human and mouse colonic epithelia (Hill et al., 2000). The hyporesponsiveness to secretagogues mediated by NO is a result of decreased adenylate cyclase activity due to this selective inhibition (Freeman and MacNaughton, 2004).

The dysfunction occurring during the acute phase of inflammation also exists in chronic inflammation. In a rat model, colitis was induced with intracolonic TNBS (Asfaha et al., 1999). Six weeks later, the colon of the TNBS-treated rats appeared macroscopically normal, but hyporesponsiveness to secretagogues remained. The decreased cAMP-dependent secretory responses were reversed with an iNOS-selective inhibitor suggesting that even after colitic tissue returns to a macroscopically normal state, ongoing iNOS expression and activity may lead to chronic epithelial secretory dysfunction (Asfaha et al., 1999). Interestingly, the NO-induced hyporesponsiveness was associated with a 3-fold increase in bacterial translocation (Asfaha et al., 2001). The bacterial translocation was prevented by daily treatment with a selective iNOS inhibitor again confirming that the increase in iNOS expression following inflammation continues even after the tissue appears macroscopically normal. In another study, the NO scavenger, NOX, was also shown to prevent bacterial translocation in rats challenged with LPS (Dickinson et al., 1999).

In a recent study using the dextran sodium sulfate (DSS)-induced model of colitis, activation of nicotinic receptors by acetylcholine caused inhibition of ion transport that was prevented by a selective iNOS inhibitor, a NO scavenger or removal of the myenteric plexus. This suggests that there is a pathway involving release of NO from neurons leading to a decrease of stimulated chloride secretion in the epithelium (Green et al., 2004).

The above studies demonstrate that increases in NO production during intestinal inflammation, specifically from increased iNOS-expression, lead to

secretory dysfunction. A direct consequence of this dysfunction is bacterial translocation. This leads to bacterial infection, and as will be discussed below, NO can also mediate the response to infection in the gut.

1.8 The Role of Nitric Oxide in Bacterial Infection:

Numerous pathogens have been studied with respect to infection in the gut. *Salmonella sp.* and *Helicobacter pylori* infections have been extensively studied and since the host epithelium uses similar strategies for a wide range of pathogens, the findings and implications from these studies could possibly be extended for other bacteria.

Studies with CaCo-2 and HT-29 colonic epithelial cells demonstrated that infection with *Salmonella sp.* could quickly and significantly up-regulate iNOS mRNA and protein expression as well as NO production (Witthoft et al., 1998). Another study demonstrated that *Salmonella sp.* infection caused increases in iNOS expression which then modulates chloride secretion and barrier function in epithelial cells suggesting that this mechanism could play a role in the diarrhea caused by the infection (Resta-Lenert and Barrett, 2002). At later stages of *Salmonella sp.* infection, it is thought that NO production can regulate the cell's apoptotic program (Kim et al., 1998). In mice infected with *Salmonella enterica*, iNOS mRNA and protein expression was increased, as was iNOS activity, within 4 hours of infection. The nitric oxide synthase inhibitor, aminoguanidine, prevented intestinal epithelial damage and villus cell apoptosis caused by *S. enterica* suggesting that iNOS is involved in the early sequelae of infection in an animal model (Giacomodonato et al., 2003). These results all implicate a role for

NO at both early and late stages in the host response to *Salmonella sp.* infection.

Helicobacter pylori is another pathogen that is known to invoke a NO response from the host epithelium. iNOS expression was found to be higher in gastric mucosa of those patients positive for *H. pylori* (Fu et al., 1999). It has also been demonstrated that NO production in murine macrophages can be stimulated by *H. pylori* infection (Wilson et al., 1996). Since host epithelial cells produce NO as an antimicrobial agent (see below), *H. pylori* has developed ways to evade this response. It has been shown that *H. pylori* contains an arginase enzyme that can consume arginine thereby down-regulating NO production by the host (Gobert et al., 2001). Further examining the mechanism of *H. pylori*-induced iNOS expression revealed that it is the urease protein released by *H. pylori* that is responsible for increases of NO (Gobert et al., 2002).

The results in the experiments involving *Salmonella sp.* discussed above provide details into the mechanisms of infection for an enteroinvasive bacterial species. Other bacteria exist that cause their effects non-invasively by attaching to the epithelial cell membrane. These pathogens are referred to as attaching and effacing pathogens due to the lesions they cause on the epithelial surface and are discussed below.

1.9 Attaching and Effacing (A/E) Pathogens:

1.9.1 Enteropathogenic (EPEC) and enterohemorrhagic (EHEC) Escherichia coli:

Enteropathogenic (EPEC) and enterohemorrhagic (EHEC) *Escherichia coli* are two of the more prominent causes of infectious diarrhea around the world (Kaper, 1998). *E. coli* is a gram-negative bacillus and certain non-pathogenic strains are found in the gastrointestinal tract of humans. EPEC is a widespread cause of infantile diarrhea in developing countries (Vallance and Finlay, 2000). EHEC is the causative agent of “hamburger disease”, causing diarrhea in adults and children including outbreaks across North America (Vallance and Finlay, 2000).

EPEC and EHEC differ from enteroinvasive pathogens in that they act non-invasively, infecting their hosts by attaching to the luminal surface of intestinal epithelial cells. This causes a rather unique morphological change which has been named an attaching and effacing (A/E) lesion. The lesion gets its name due to the pathogen attaching to the epithelial cell surface and the resulting effacement of brush border microvilli.

1.9.2 LEE, Type III Secretion, Virulence Factors:

In order for EPEC and other A/E pathogens to cause a change in the host, it is necessary for virulence factors to cross the epithelium. EPEC acts as a non-invasive extracellular pathogen, so these factors are delivered to the host through a specialized Type III secretion system. This system consists of approximately 20 protein components and is highly conserved among pathogenic

strains of gram-negative bacteria. The genes which encode the proteins for the Type III secretion system are grouped on chromosomal pathogenicity islands. The locus for enterocyte effacement (LEE) is a pathogenicity island in EPEC and it is necessary and sufficient for A/E lesion formation (McDaniel et al., 1995).

The open reading frames in the LEE are organized into five operons. Three of these operons encode the proteins required for the structural subunits of the Type III secretion system. The fourth operon encodes the genes required for attachment of the bacteria to the host epithelial cell. These are *eae*, *tir*, and *cesT*. The fifth operon contains the *esp* (*E. coli* secreted protein) genes that are secreted by the Type III secretion system. (Donnenberg and Whittam, 2001)

1.9.3 A/E Pathogen Epithelial Interactions and Pedestal Formation:

A/E lesions can be as high as 10 μm above the host cell (Moon et al., 1983) and their development is often referred to as pedestal formation. Many advances have been made in recent years in studies aimed at better characterizing the pathogenesis of EPEC. Adherence of *E. coli* initiates via pili which are able to attach to the microvilli on the cell surface (Moon et al., 1983). The microvilli then disappear from the cell surface allowing the *E. coli* to make even closer contact. The bacteria then uses the Type III injector system to introduce bacterial proteins into the cell. EspA projects from the bacterium to the cell surface and forms part of the filamentous bridge between the bacteria and the host cell, an event which precedes the formation of A/E lesions (Knutton et al., 1998). EspB and EspD form an opening in the cell membrane allowing for more bacterial proteins to move within the cell (Vallance and Finlay, 2000). It

has also been suggested that the extracellular domain of EspD, once within the host cell membrane, may interact with EspA containing filaments (Fivaz and van der Goot, 1999). The translocated intimin receptor (Tir) is then inserted in the cell membrane and binds to intimin on the bacterial cell surface making the bacterium completely bound to the intestinal cell (Kenny and Finlay, 1997). For A/E lesions to form, a tyrosine residue on Tir must be phosphorylated (Kenny, 1999). Cytoskeletal proteins from the intestinal cell bind to Tir that is within the cell and actin strands are formed. As these filaments become longer, the cell membrane is pushed up directly under the bacterium and a pedestal for the bacterium is formed (Knutton et al., 1989). The formation of pedestals is caused directly by rearrangement of host cell cytoskeletal proteins. Intimin is a protein in the outer membrane of the bacterial cells. It is thought that intimin binds and clusters Tir and that this event is responsible for triggering the host cell to form pedestals under the bacteria adhered to the surface (Goosney et al., 2000).

Other translocated proteins have been identified and studied in terms of pedestal formation in the past few years. Some examples include EspF, EspG and mitochondrial-associated protein (MAP). They do not appear to play a role in pedestal formation itself, although their precise roles remain unresolved. EspF mutants were shown to impair EPEC's ability to disrupt barrier function compared to wild-type EPEC (McNamara et al., 2001), but the role of EspF in an animal model has yet to be investigated. The role of EspG has been studied in a rabbit model of REPEC infection (Elliott et al., 2001). In these studies, an EspG mutant strain caused delayed intestinal colonization suggesting that EspG may play a

role in the initial interaction between the pathogen and the epithelium. In vitro studies have shown that EspG can interact with the host cytoskeleton leading to microtubule destruction (Hardwidge et al., 2005). MAP is able to target the host mitochondria and disrupt its function in a manner that in synergistic with Tir as demonstrated by EPEC strains lacking MAP, Tir, or both (Jepson et al., 2003).

While the mechanisms of attachment of A/E pathogens have been studied in detail, there is still the need to extend beyond an *in vitro* system and investigate the mechanisms leading to tissue damage and disease. This includes further study into the host immune response following formation of A/E lesions. One difficulty in bringing these studies into a host system is that while EPEC and EHEC cause disease consistently in humans, rabbits and gnotobiotic piglets, this pathogen is not specific to mice. Mouse models are useful for studying host-pathogen interactions due to the availability of animals with specific gene deficiencies. While it has been shown recently that EPEC is capable of infecting mice (Savkovic et al., 2005), *C. rodentium* is a mouse-specific pathogen providing a reproducible model of A/E pathogen infection.

1.10 Citrobacter rodentium:

Citrobacter rodentium, originally named *Citrobacter freundii* biotype 4280, is the causative agent of transmissible murine colonic hyperplasia (TMCH) (Barthold et al., 1976), which occurs naturally in laboratory mice (Barthold et al., 1978; Schauer et al., 1995). This disease is characterized by epithelial cell hyperproliferation in the descending colon (Barthold et al., 1978). Early studies

made the observation that *C. rodentium* colonizes colonic mucosa and are capable of producing A/E lesions (Johnson and Barthold, 1979) that are indistinguishable from those produced by EPEC and EHEC in humans (Schauer and Falkow, 1993a). The protein responsible for attachment, intimin, is functionally homologous in EPEC and *C. rodentium*. The system required for A/E lesion formation is regulated by the LEE pathogenicity island. There is strong homology between the LEE genes in pathogenic strains of *E. coli* and the mouse pathogen *C. rodentium* (Deng et al., 2001). These characteristics provide the basis for a small animal model of A/E pathogen infection resembling EPEC and EHEC infection of the human gut.

In addition to crypt hyperplasia, *C. rodentium* also causes inflammatory cell infiltration including macrophage, lymphocytes, and neutrophils (Barthold et al., 1976; Barthold et al., 1978; Schauer and Falkow, 1993b; Higgins et al., 1999). Mice generally clear the infection within 3-6 weeks and are immune from a second infection (Ghaem-Maghami et al., 2001). Infection of mice with *C. rodentium* does not generally lead to significant diarrhea, morbidity or mortality (Schauer and Falkow, 1993a).

The immune response to *C. rodentium* infection has been studied recently with respect to clearance of infection. It was found that B cells and IgG, but not IgA or IgM antibodies are required for host defense against *C. rodentium* (Maaser et al., 2004). Indeed, mice lacking B cells were not able to clear the bacterial infection over an extended time period (Maaser et al., 2004; Simmons et al., 2003). It was interesting that secretory antibodies in the lumen were not

required for *C. rodentium* clearance since it is at the epithelial surface that the majority of the bacteria are located. However, other host defense mechanisms, such as NO from iNOS (Vallance et al., 2002) or defensins (Wilson et al., 1999; O'Neil et al., 1999), may play a role in controlling infection.

While the immune response to *C. rodentium* has been studied in some detail, relatively little is known about epithelial function during or following infection. To address this lack of knowledge is important, given the need to better understand the mechanisms underlying infection-associated pathology, and to determine the role that bacterial pathogens play in exacerbation of existing inflammatory conditions.

1.11 Radiation and its Biological Effects:

In addition to damage caused to the intestinal epithelium by invading pathogens, other factors such as ionizing radiation have also been shown to cause intestinal inflammation. The destructive and mutagenic effects of radiation were clearly observed following the 1986 nuclear accident in Chernobyl (Bard et al., 1997). However, radiotherapy is also useful in a therapeutic setting, but can lead to significant biological effects. As will be discussed below, intestinal complications such as bacterial translocation and overgrowth can occur following ionizing radiation treatment. Therefore, a better understanding of these effects will prove helpful in the therapeutic setting.

Radiation is defined as the transmission of energy and is generally characterized based on its reactions with other matter. For instance, radiation

can be classified as non-ionizing or ionizing according to its energy. Radiation can be measured in different units although the clinically used unit is the Gray (Gy). 1 Gy is defined as 1 Joule of energy absorbed in each kilogram of absorbing material (Mettler and Moseley, 1999). Ionizing radiation is used therapeutically, and therefore its biological effects are the focus of this section.

Although all doses of radiation may cause damage to biological systems, the damage that is caused is proportional to the dose received. Doses of less than 1 Gy can cause leukemia and tumours within a time frame of 3-10 years. At higher doses, more immediate complications can occur. Hematopoietic system damage and death can occur within a month at a dose of 10 Gy. Doses between 10-40 Gy can cause gastrointestinal damage leading to death within days. At high doses (>50 Gy), CNS damage occurs leading to death within hours (Berry and Denekamp, 1989).

The deleterious effects of radiation begin with DNA damage (Wallace, 1998). DNA can be damaged in a number of ways including nucleotide base damage and single or double-stranded breaks. DNA damage, if not repaired, will have downstream effects on intracellular events and cell cycle arrest. When enough cells are damaged, these effects become manifested at the tissue level. The fact that these events occur predominantly in actively dividing cells makes radiation an effective means of arresting tumour growth. While this underlies its therapeutic efficacy, it also renders healthy, rapidly dividing tissues, such as the gastrointestinal epithelium, susceptible to damage. Indeed, one major problem in patients undergoing abdominopelvic radiotherapy is the development of

radiation-induced gastrointestinal symptoms including epithelial dysfunction (Kao, 1995). While mechanisms of acute radiation injury are well described, the link between epithelial dysfunction following irradiation and development of chronic intestinal disease is still not understood.

It has also been known for some time that intestinal exposure to ionizing radiation causes bacterial translocation (Guzman-Stein et al., 1989; Rosoff, 1963). The precise mechanism by which the bacteria are able to cross from the lumen into the lamina propria is not known, but it has been shown that the bacterial translocation is related to the expression and activity of iNOS. In rats challenged with LPS, the NO scavenger NOX significantly reduced the incidence of bacteremia and transmucosal passage of bacteria through ileal membranes mounted in Ussing chambers (Dickinson et al., 1999). The NO synthase inhibitor aminoguanidine was also shown to significantly reduce bacterial translocation in the same experimental design (Sorrells et al., 1996). In other studies using iNOS-deficient mice, bacterial translocation did not occur following exposure to endotoxin (Mishima et al., 1997). While the above has provided evidence for an increase in iNOS expression and bacterial translocation following ionizing radiation, it is not known if the increase in iNOS expression is directly responsible for the increase in bacterial translocation.

Previous studies in our lab have focused on the acute phase of epithelial dysfunction (Freeman and MacNaughton, 2000; Freeman and MacNaughton, 2004). These studies involved exposing the whole mouse to the gamma-radiation source. One downside to this type of treatment is the increase in

morbidity and mortality following irradiation. However, since it is generally accepted that acute affects of irradiation can lead to chronic complications, studying the acute phase following irradiation still provides useful insight. As the mechanisms of epithelial dysfunction are further elaborated, moving into a chronic model of irradiation may also prove useful. A model has been reported (Skwarchuk and Travis, 1998) looking at a model of chronic radiation enteritis in C57Bl/6 mice and can therefore serve as a model to further study the effects of high dose irradiation on the abdominopelvic region alone.

1.12 Summary:

In summary, the multiple roles played by the intestinal epithelium results in a complex interface with the external environment. The epithelium cannot simply act as an impermeable barrier, since it is also the site of action for nutrient and water absorption. Epithelial dysfunction may lead to inflammatory diseases such as inflammatory bowel disease or radiation enteritis. In both of these conditions, a luminal pathogen is at least partially involved in the development of inflammatory symptoms.

The secretion of ions, chloride in particular, is important in maintaining epithelial integrity as it creates osmotic pressure leading to secretion of water acting to flush out pathogens and antigens. While the function of chloride channels, such as CFTR, are well described in healthy conditions, inflammation can lead to mediators such as NO being produced, and these have effects on secretory function. We have previously shown that NO inhibits cAMP-dependent chloride transport in epithelial cells but the mechanism of this is not yet clear.

The trafficking of CFTR to the apical membrane of epithelial cells is known to be cAMP-dependent in normal functioning cells. Whether NO can affect CFTR trafficking directly and account for the NO-dependent decrease in chloride transport is not known.

In addition to affecting the secretion of chloride, in inflammatory conditions, NO may also play a role in cell permeability and subsequent bacterial translocation. While other studies have investigated NO in these settings, the role of NO in our cell model system has not yet been studied with respect to tight junction proteins and epithelial permeability.

Moving from a cell line model to an animal model is an important step for investigating the role of inflammation in general, and NO in particular, in a whole body system. Bacterial infection and translocation has been shown to induce an inflammatory response. Many studies using the *C. rodentium* model of A/E pathogen and epithelial interaction have focused on the mechanisms underlying the development of the inflammatory response to *C. rodentium* infection. However, little is known about the effect of infection on mucosal barrier and ion transport function.

It has long been known that exposure to ionizing radiation leads to bacterial translocation. However, the mechanisms for this have not been fully resolved. In this study, we use a mouse model of irradiation combined with *C. rodentium* infection to examine the effects on epithelial dysfunction and bacterial translocation.

A better understanding of the functions and mechanisms in intestinal inflammation involving secretory dysfunction and bacterial translocation will provide clinical insights and strategies aimed at preventing disease.

1.13 Hypothesis and Objectives:

Nitric oxide inhibits intestinal epithelial chloride secretion, thus allowing bacterial translocation which contributes to enteritis following irradiation.

This research project will attempt to gain a better understanding of the role of nitric oxide with respect to chloride secretion and models of inflammation. This study has implications for diseases involving changes in barrier function and secretion including infectious colitis and radiation enteritis. The objectives formed to test the hypothesis were:

1. To determine the role of nitric oxide in CFTR trafficking and chloride secretion in epithelial cells. In these studies, epithelial cells were treated with a nitric oxide donor and CFTR trafficking and secretory function were assessed.
2. To determine the role of nitric oxide in tight junction integrity, permeability and bacterial translocation in epithelial cells. Epithelial cells were treated with nitric oxide and tight junction proteins were assessed for disruption. Permeability of epithelial cell monolayers was also measured following treatment with a nitric oxide donor.

3. To characterize epithelial dysfunction following *Citrobacter rodentium* infection. Mice were infected with *C. rodentium* and assessed for functional changes in mucosal barrier and ion transport function. The role of NO in *C. rodentium*-induced mucosal dysfunction was determined.
4. To determine the role of the combined actions of *Citrobacter rodentium* infection and ionizing radiation. Mice were infected with *C. rodentium* and exposed to ionizing radiation to assess mucosal barrier and ion transport function.

Chapter 2: General Methods and Materials:

2.1 Experimental Models:

2.1.1 SCBN:

SCBN cells are a non-transformed duodenal epithelial cell line that grows as high-resistance polarized monolayers and are capable of apical chloride secretion. SCBN cells behave as crypt-like epithelial cells and therefore provide a model for the study of the mechanisms underlying chloride secretion (Pang et al., 1996). The cells were originally cultured from endoscopically obtained biopsies of the duodenum of a patient presenting with diarrhea of unknown etiology and were originally referred to as human duodenal crypt-like cells. Although, the karyotype of these cells is unusual, they are very easy to grow and have served as a useful model for studying epithelial chloride transport (Buresi et al., 2001).

SCBN cells between passages 25 and 35 were grown in culture flasks or on Transwell membranes (Costar) by methods previously described (Pang et al., 1996; Dharmsathaphorn et al., 1984; Augeron and Laboisse, 1984). SCBN cells were grown in Dulbecco's Modified Eagle Medium supplemented with 5% fetal bovine serum, L-glutamine, tylosin, and penicillin-streptomysin. When confluent, as determined by light microscopy, cells were passaged using 1.5X trypsin-EDTA and were fed every 2-3 days with the media described above. SCBN cells were seeded onto Transwell membranes 48 hours before experiments allowing for adhesion to the wells. For Ussing chamber experiments, SCBN cells were grown on Snapwell supports (Costar) and only monolayers with resistances of greater

than 1000 Ohms/cm^2 measured using an electrovoltohmmeter (EVOM, World Precision Instruments, Sarasota, FL) were used.

In some experiments, SCBN cells were grown on Labtec chamber slides. Before seeding the cells, chamber slides were coated with collagen to facilitate better adhesion of the cells to the slide. Media was changed daily until cells reached confluence as determined by light microscopy.

2.1.2 Mice

Male C57Bl/6 mice (Charles River, Montreal, PQ) were used for all experiments unless otherwise noted. All mice were 4-6 weeks of age upon arrival and were allowed to acclimatize for at least 5 days prior to beginning experiments. The mice were housed in the Biohazard Facility of the University of Calgary Animal Resources Centre and were given free access to standard chow and tap water. All mouse experiments were approved by the University of Calgary Animal Care Committee and followed the established guidelines of the Canadian Council on Animal Care.

Experiments involving iNOS-deficient mice (Jackson Labs, Bar Harbor, ME) used strain B6.129Ps-Nos2^{tm1lau} (10 backcrosses). The wild-type mice for these experiments were of the same background strain (C57Bl/6) and were obtained from the same source as the iNOS-deficient mice.

In experiments involving bacterial infection, *C. rodentium*, strain DBS 100, was a generous gift from Dr. David Schauer (MIT, Cambridge, MA) and has been previously characterized as a murine pathogen (Luperchio et al., 2000). The

bacteria were grown under aerophilic conditions on MacConkey agar plates overnight at 37^0C. Bacteria were then harvested and grown in Penassay broth overnight at 37^0C. Mice were orogastrically infected with 10^8/mL live bacteria in 0.1 mL broth as determined by the growth curve for *C. rodentium*. Sham controls received an equal volume of sterile broth alone. The day prior to euthanasia of the mice, rectal swabs were plated onto Columbia SB blood agar (Becton, Dickinson and Company, Sparks, MD) and MacConkey agar plates (PML Microbiologicals, Mississauga, ON) to confirm colonic colonization of gram-negative lactose fermenting bacteria. These colonies were easily identified by their size and appearance (Vallance et al., 2002).

2.2 Immunofluorescence:

For experiments involving CFTR trafficking, cells were grown on Transwell membranes as described above, and then prepared for immunofluorescence of CFTR. The following procedure took place within the wells attached to the Transwell membranes. Cells were treated, washed, and then fixed in cold methanol for 20 minutes at -20°C. The fixed cells were washed 3 times with Dulbecco's phosphate buffered saline (PBS; 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 8.0 g/L NaCl, and 1.15 g/L Na_2HPO_4), then permeabilized in 0.1% Triton-X in PBS for 2-5 minutes. The 0.1% Triton-X in PBS was removed and blocking solution added consisting of 5% BSA and 10% goat serum in 0.1% Triton-X in PBS. The blocking solution was removed and the primary antibodies added. Negative controls and preparations without primary antibodies were used as controls. The

primary antibodies consisted of monoclonal anti-human CFTR (R domain specific) antibody (R&D Systems) at 1:100 and rabbit anti-ZO-1 (Zymed, Eugene, OR) at 1:100. ZO-1 staining was used to visualize tight junctions and was used as a marker to distinguish between apical and basolateral membranes. The negative controls were mouse IgG1 negative control (Dako) and preimmune rabbit serum. The antibodies were diluted in blocking solution and applied to the cells for 1 hour. The cells were then washed 5 times with PBS and secondary antibodies were applied for 1 hour. The secondary antibodies consisted of goat-anti-mouse-Cy3 (1:100; Jackson) to visualize the CFTR and goat-anti-rabbit-FITC (1:100; Molecular Probes) to visualize ZO-1. The cells were then washed 5 times in PBS.

Upon completion of the staining procedure, the Transwell filters were cut from the wells and embedded on edge in paraffin to allow for cross-sectional visualization of the cell monolayers. The samples were left in paraffin overnight and cut the next day in 5 μm sections using a microtome. The sections were mounted on silane treated slides for better adhesion and placed on a slide warmer overnight. The slides were then deparaffinized using NeoClear (EM Sciences), re-hydrated, and coverslipped using FluorSave Reagent (Calbiochem) as a mounting medium.

For experiments involving the fluorescent staining of tight junction proteins, cells were grown on chamber slides as described above. Primary antibodies in addition to ZO-1 included anti-mouse claudin-4, claudin-5 and

occludin (all available from Zymed, Eugene, OR) used at a dilution of 1:250. The secondary goat-anti-mouse-Cy3 (Jackson) was used at a dilution of 1:1000.

In CFTR experiments, cross-sections were visualized using a Deltavision Deconvolution microscopy system (Applied Precision LLC, Issaquah, WA). In experiments visualizing tight junction proteins of cell monolayers, slides were viewed using a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany), images taken with a Sensys digital camera (Photometrics, Tucson, AZ) and screen captured using V for Windows imaging software (Digital Optics, Aukland, NZ). The settings for each microscope are described in the relevant sections below.

2.3 Measurement of Chloride Secretion:

Chloride secretion in SCBN cells was measured by growing the cells on Snapwell semipermeable supports (VWR, Mississauga, ON). The Snapwells were mounted in modified Ussing chambers (Harvard Apparatus Canada, Saint-Laurent, QC) and bathed in Kreb's buffer (115 mM NaCl, 2 mM KH_2PO_4, 2.4 mM $MgCl_2$, 25 mM $NaHCO_3$, 8 mM KCl, and 1.3 CaCl). Kreb's buffer containing 10 mM mannitol was added to the apical side and Kreb's buffer containing 10 mM glucose was added to the basolateral side. In mouse experiments, mice were killed by cervical dislocation and proximal colons were mounted in Ussing chambers and bathed on both sides with Kreb's buffer containing glucose. The buffer was bubbled with 5% CO_2, 95% O_2 and were maintained at a temperature of 37^{o}C and pH 7.4. In addition to providing oxygen to the cells and tissue, the

bubbling was also used to circulate the buffer within each compartment allowing for drugs to reach the cells or tissue.

Using a VCCMC8 voltage clamp apparatus (Physiologic Instruments, San Diego, CA), a short circuit current was applied to clamp the transepithelial voltage to zero. Current was delivered and potential difference was measured using an EasyMount electrode set (Physiologic Instruments, San Diego, CA). The changes in short circuit current (ΔIsc) reflected changes in the net electrogenic ion flux across the monolayer. Isc was recorded by a digital data acquisition system (MP100, BioPac Inc., Goleta, CA) and analyzed using AcqKnowledge 3.5.7 software (BioPac). Isc was normalized to 1 cm^2 tissue surface area.

2.4 Intestinal Inflammation:

2.4.1 Macroscopic Damage Score and Wall Thickness:

Mice were killed by cervical dislocation. Following laparotomy, colons were removed and cut open along the mesenteric border. Macroscopic damage was scored according to the following parameters. 1 point each was given for presence of erythema, hemorrhage, edema, stricture formation, ulceration, fecal blood, mucus presence, diarrhea, and adhesions. An extra point for each was given if erythema or adhesions were severe. Colonic thickness was determined using Absolute Digimatic digital calipers (Mitutoyo Corporation, Japan) and was expressed in mm.

2.4.2 Myeloperoxidase Activity:

Myeloperoxidase activity, a measure of granulocyte infiltration into the tissue, was determined as previously described (Wallace et al., 1992). Briefly, 35-100 mg of full thickness distal colonic tissue was homogenized with a Polytron PT 10/35 homogenizer (Kinematica AG, Littau-Lucerne, Switzerland) in 5% hexadecyltrimethylammonium bromide phosphate buffer. Following centrifugation at 13,000 x *g* at 4^{o}C for 3 minutes, the supernatant was removed. Oxygen produced by peroxidation of H_2O_2 reacts with *o*-dianisidine (200 μL) to produce a coloured product. Peroxidase activity was measured colorimetrically by determining the optical density of the reaction product 6 times at 2 min intervals, at 450 nm using a Spectromax Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) and SoftMax Pro 3.0 software (Molecular Devices).

2.5 Bacterial Translocation:

To determine bacterial translocation, mesenteric lymph nodes, liver, and spleen were removed and homogenized in sterile Dulbecco's phosphate-buffered saline (PBS). Homogenates were plated onto MacConkey agar, incubated overnight at 37^{o}C, and bacterial colony-forming units (CFU) enumerated. The frequency of bacterial translocation was expressed as the proportion of mice showing positive lymph node, spleen or liver colonization. For the studies involving rofecoxib treatment, fecal pellets were also collected, homogenized and plated on MacConkey agar to determine whether rofecoxib treatment had an

effect on the colonic microflora. CFU were counted and expressed as CFU/mg fecal pellet.

2.6 Western Blot:

Distal colonic tissue, 35-100mg, was taken from mice killed 7, 10 and 60 days after sham or *C. rodentium* infection for examination of iNOS and COX-2 expression by Western blotting. The tissue samples were homogenized in lysis buffer (0.1% Triton X-100, 50 μM Pepstatin-A, 0.2 mM Leupeptin, 1 μg/ml aprotinin, 10 mg/ml phenylmethyl sulfonyl fluoride, 50 mM Tris, 10 mM EDTA). The samples were then centrifuged and the protein concentration of the supernatant was determined by colorimetric protein assay (BioRad, Hercules, CA). Protein (30 μg) was separated on a 10% polyacrylamide gel (150 V) and then transferred (300 mA, 1 hour; BioRad Blotter, Hercules, CA) to a nitrocellulose membrane. The membrane was incubated for 1 h with blocking buffer (20 mM Tris, 100 mM NaCl, 0.5% Tween 20, and 5% non-fat dried milk) and then probed overnight at 4^0C with antibody against β-actin (control) , iNOS (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) or COX-2 (1:500; Cayman Chemical, Ann Arbor, MI). The membrane was then incubated with an appropriate peroxidase-conjugated secondary antibody for 1 h at room temperature. ECL chemiluminescence reagent (Amersham Biosciences, Baie d'Urfé, PQ) was added to visualize the labeling according to the manufacturer's instructions. Densitometry was conducted using a GS-710 Calibrated Imaging

Densitometer (BioRad, Hercules, CA) and analyzed with Quantity One software (BioRad, Hercules, CA).

2.7 Materials:

All components for buffers were obtained from Sigma-Aldrich (Oakville, ON), BDH Incorporated (Toronto, ON) or EM Sciences (Gibbstown, NJ). Unless otherwise stated, all other experimental materials were obtained from Sigma-Aldrich (Oakville, ON).

2.8 Statistics:

Data are expressed as the mean ± standard error of the mean. Statistical comparisons were made using GraphPad Instat software (version 3.0, GraphPad Software, San Diego, CA). For studies with more than two groups, one-way analysis of variance with a *post hoc* Tukey test was used. Studies with two groups were compared using an unpaired two-way Student's *t*-test. For comparison of damage scores, groups were compared using a non-parametric Mann-Whitney test. A probability (*P*) value of <0.05 was considered significant.

Chapter 3: Role of Nitric Oxide in CFTR Trafficking in Epithelial Cells:

3.1 Introduction:

The transport of fluid in the gastrointestinal tract is achieved through osmotic gradients created by the vectorial movement of electrolytes and other solutes. Chloride secretion is considered the predominant process involved in luminally directed fluid movement (Barrett and Keely, 2000). Secretion of chloride and, hence, water is considered part of the host defense function of the intestinal epithelium and serves to prevent the translocation of bacteria, bacterial products and antigens from the lumen to the lamina propria. This secretory component of the epithelial barrier is disrupted in inflammatory diseases of the intestine as shown in animal models of colitis (MacNaughton et al., 1998; Asfaha et al., 1999) and radiation enteropathy (Freeman and MacNaughton, 2000) and in human disease (Archampong et al., 1972; Hubel and Renquist, 1990; Sandle et al., 1990). In these cases, the epithelium becomes unresponsive to secretagogues, even after the inflammatory episode has resolved (Asfaha et al., 1999). Recently, we demonstrated that nitric oxide (NO), derived from the inducible isoform of NO synthase (iNOS) inhibits cAMP-dependent chloride secretion during or following a bout of colitis (MacNaughton et al., 1998; Asfaha et al., 1999) and that this leads to an increase in bacterial translocation across the intestinal epithelium (Asfaha et al., 2001). Furthermore, we have shown that the effect is likely due to the inhibition of adenylate cyclase isoform 5 and/or 6 (Freeman and MacNaughton, 2004), which mediate, in part, cAMP-dependent

secretion and which have been shown to be sensitive to NO (Hanoune and Defer, 2001).

The cystic fibrosis transmembrane conductance regulator (CFTR) is a key protein involved in ion channel regulation and chloride secretion in several epithelia, including the intestine (Barrett and Keely, 2000). Regulation of CFTR-mediated chloride secretion occurs through three processes: phosphorylation leading to channel opening, trafficking of CFTR to the membrane, and CFTR endocytosis. The control of these processes is complex and has been recently reviewed (Bertrand and Frizzell, 2003). Each process has a component that is dependent upon cAMP and the activation of protein kinase (PK) A (Bradbury, 2001). The role of cAMP in the gating function of apical membrane-bound CFTR has been well described (reviewed in Gadsby and Nairn, 1999). Similarly, trafficking of CFTR from cytoplasmic stores to the apical membrane has been described, particularly as it relates to cystic fibrosis. The ΔF508 mutation responsible for upwards of 90% of CFTR mutations resulting in cystic fibrosis (Mickle and Cutting, 1998) is a case in point. With this mutation, trafficking of CFTR to the apical plasma membrane is impaired (Puchelle et al., 1992) and chloride secretion is thus reduced, resulting in disease. Trafficking of wild-type CFTR to the apical membrane appears to be a cAMP-dependent process, as T84 cells stimulated with the adenylate cyclase activator forskolin exhibit increased CFTR immunoreactivity at the apical surface (Tousson et al., 1996) and inhibition of trafficking is associated with the blockade of the cAMP-induced increase in CFTR-mediated chloride secretion (Schwiebert et al., 1994).

While we have shown that iNOS-derived NO inhibits cAMP-dependent chloride transport by intestinal epithelial cells, we have not determined whether this is an effect on phosphorylation of CFTR present in the apical plasma membrane or on CFTR trafficking. Based on the observation that increases in intracellular cAMP stimulated insertion of CFTR into the apical plasma membrane of T84 colonic epithelial cells (Schwiebert et al., 1994; Tousson et al., 1996), we have tested the hypothesis that nitric oxide inhibits cAMP-dependent CFTR translocation into the apical plasma membrane, thereby reducing chloride secretion, using the SCBN crypt cell line.

3.2 Methods:

3.2.1 Cell Line:

SCBN cells were used in these studies. Cells (passages 25-35) were grown to confluence in culture flasks, Transwell membranes, or Snapwell membranes according to the methods in Chapter 2.1.1.

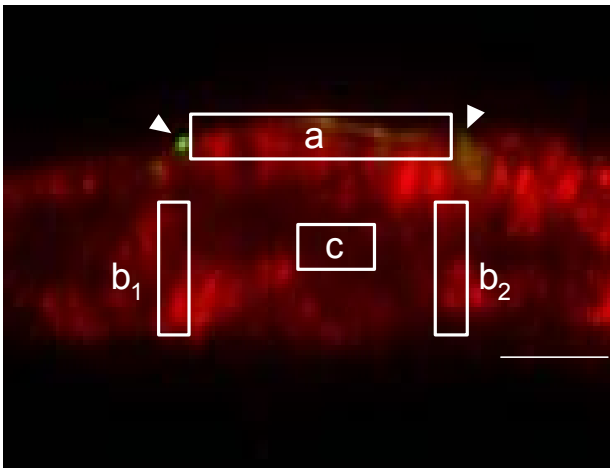
3.2.2 Immunofluorescence:

Cells were grown on Transwell membranes and immunofluorescence for CFTR was performed as described in Chapter 2.2. The fixed, permeabilized cell cross-sections were visualized using fluorescent microscopy on a Delta-Vision deconvolution microscopy system (Applied Precision LLC, Issaquah, WA). The CFTR was visualized using a rhodamine filter. The ZO-1 was visualized using a FITC filter. Images were taken using a 100X objective, and adjacent pixels averaged with a bin setting of 2 to allow for clearer images. The apical to

basolateral ratio of CFTR immunofluorescence was measured using a modification of the procedure described by Moyer and Stanton (Moyer and Stanton, 2002) as shown in Figure 3.1. Briefly, the apical membrane was identified using immunofluorescence for the tight junction protein ZO-1 which also provided the boundary for each cell. The nuclear compartment was identified using bis-benzimide (0.5 mg/mL; 1:500 dilution) and visualized using a DAPI filter. Light microscopy images were also used to very the size and location of the cells on the Transwell filters.

3.2.3 Measurement of chloride secretion:

Chloride secretion in SCBN cells was measured by growing the cells on Snapwell semipermeable supports (VWR, Mississauga, ON) as described in Chapter 2.3. An electrovoltohmmeter (World Precision Instruments, Sarasota, FL) was used to measure resistance across the monolayers to determine confluency. Only monolayers with resistances greater than 1000 Ohms/cm^2 were used. Monolayers were stimulated using 10 μM forskolin added to the basolateral side of the monolayers. The nitric oxide donor PAPA-NONOate was also added to the basolateral side of the monolayers. Nocodazole was added to both the apical and basolateral side of the monolayers.



$$R = \frac{(a - c)}{[(b_1 - c)/2 + (b_2 - c)/2]}$$

Figure 3.1. Measurement of the apical:basolateral ratio of fluorescence in cross-sections of monolayers of SCBN cells, based on the method of Moyer and Stanton (Moyer and Stanton, 2002). Apical and basolateral membranes were demarcated by immunostaining for ZO-1 (green fluorescence, arrowheads). Intensity of CFTR fluorescence (red) was measured in the apical (a), basolateral (b) and nuclear (c) compartments. The ratio of apical:basolateral fluorescence (R) was calculated according to the formula shown. The white bar represents the interface between the bottom of the cells and the filter on which they were grown.

3.2.4 cAMP Concentration Determination:

Cells were grown in 75 cm^2 flasks to confluence as determined by light microscopy. Cells were washed with sterile phosphate-buffered saline and 4 ml FBS- and anti-biotic free DMEM was added to each flask. Cells were lifted using a cell scraper. Cell suspensions were aliquoted (1 ml) into microfuge tubes and placed in a shaker water bath at 37^oC. 100 μM PAPA-NONOate or the vehicle was added to each tube. After 20 minutes, the phosphodiesterase inhibitor, B-8279 (Sigma; 100 μM) was added followed immediately by 10 μM forskolin or vehicle. After incubating for 5 minutes, the microfuge tubes were placed in liquid nitrogen. The cells were lysed by cycling the tubes between liquid nitrogen and a 40^oC water bath 3 times. Tubes were then centrifuged for 5 minutes at 15,000 *g* at 4^oC. The concentration of cAMP in the supernatants of each tube was determined using a commercially available ELISA kit (Cayman Chemical, Ann Arbor, MI) and expressed as pmol/ml.

3.2.5 Materials:

PAPA-NONOate (Sigma-Aldrich, Oakville, ON) is a NO donor compound with a half-life of 20 minutes at 37^oC and pH 7.4. It dissociates 2 moles of NO for each mole of parent compound. A dose of 100 μM was chosen since it did not completely block chloride secretion allowing for study of the combined effects with nocodazole.

All drugs were purchased from Sigma-Aldrich (Oakville, ON) unless otherwise stated. Routine chemicals for the preparation of buffers were purchased from BDH Inc (Toronto, ON).

3.3 Results:

Basolateral application of forskolin to SCBN monolayers mounted in Ussing chambers resulted in a rapid increase in short circuit current. The Isc change in response to forskolin peaked between 60-90 seconds. In control monolayers fixed in methanol and processed for immunocytochemistry, CFTR-immunoreactivity was evenly dispersed throughout the cytoplasm and the apical:basolateral ratio of immunofluorescence was approximately 1 (Figure 3.2). However, as early as 30 s following forskolin stimulation, CFTR immunofluorescence appeared more intense in the apical plasma membrane. This was reflected in a statistically significant increase in the apical:basolateral fluorescence ratio at 30 s and 2 min after addition of forskolin to the bath (Figure 3.2).

In Ussing chamber experiments, addition of forskolin caused a rapid increase in Isc that was statistically significant at 30 s, 2 min and 4 min when compared to the zero time point (Figure 3.2). At 10 min, the forskolin-induced change in Isc was not significantly different from the zero time point. The peak change in the apical:basolateral CFTR fluorescence ratio occurred at 30 sec and preceded the peak change in Isc, which observed at the 2 min time point (Figure 3.2).

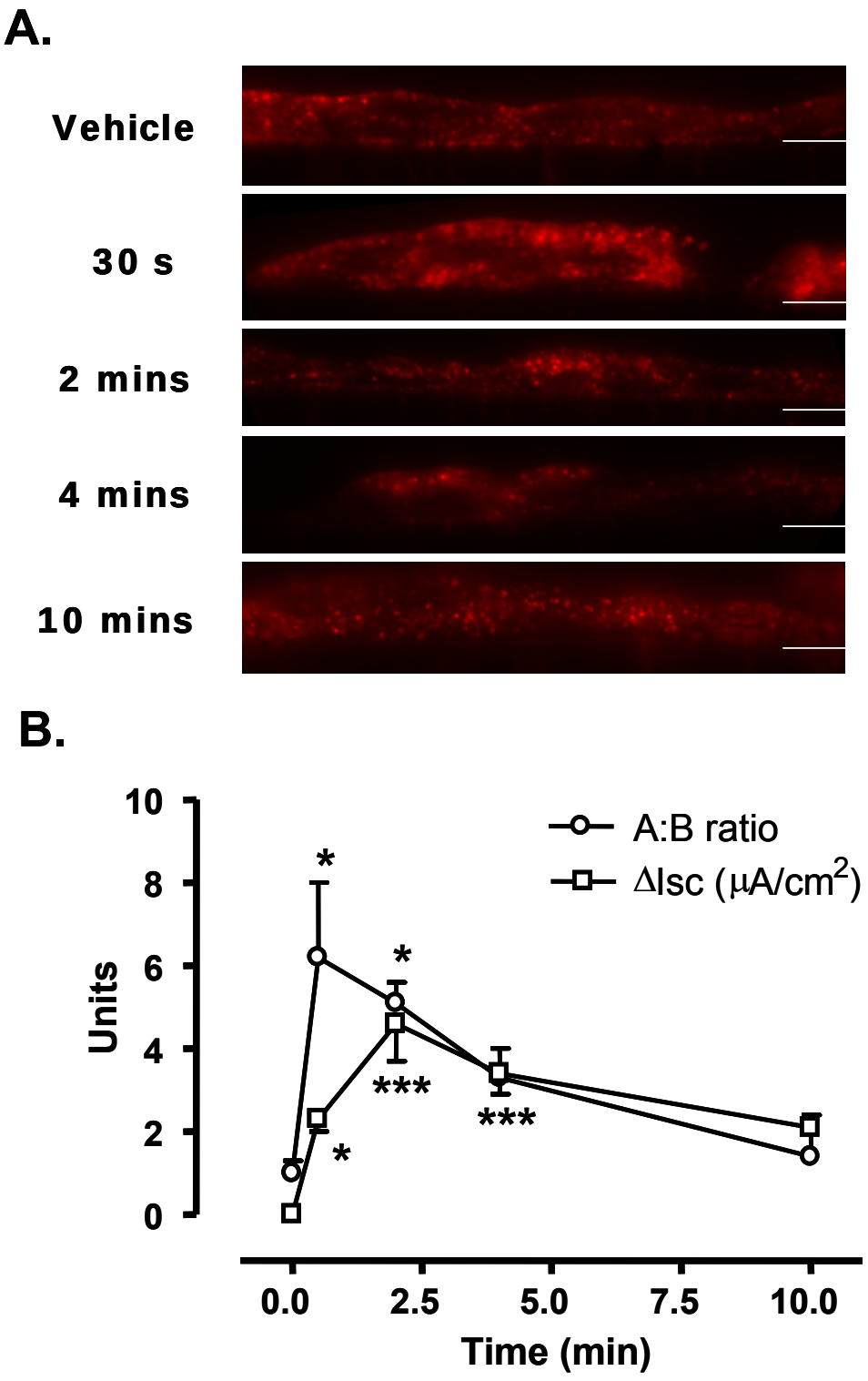

A.
Vehicle
30 s
2 mins
4 mins
10 mins
B.
A:B ratio
ΔIsc (μA/cm2)
Units
10
8
6
4
2
0
*
*
*
***
***
0.0
2.5
5.0
7.5
10.0
Time (min)

Figure 3.2.

Figure 3.2. CFTR fluorescence in SCBN monolayers at various times after exposure to forskolin (10 μM). A. Representative micrographs of CFTR immunofluorescence in control (vehicle) monolayer and at 30 s and 2, 4 and 10 min after addition of forskolin. The white bar represents the interface between the bottom of the cells and the filter on which they were grown. B. Graph of the ratio of apical:basolateral CFTR fluorescence (open circles, n=6-8) and short circuit current (ΔIsc, open squares, n=7-20) following exposure to 10 μM forskolin on the basolateral side. *p<0.05, ***p<0.001 vs. vehicle (0.0 min).

To test the mechanism leading to the increase in apical to basolateral CFTR immunofluorescence, the PKA inhibitor H89 was used. Following pretreatment with 5 μM H89, 10 μM forskolin stimulation for 2 minutes caused a significant decrease in fluorescence ratio compared to cells treated with forskolin alone (Figure 3.3). H89 treatment in the absence of forskolin did not cause a change in the apical to basolateral fluorescence ratio.

To determine if NO also decreases the CFTR trafficking response to forskolin, NO donor PAPA-NONOate (100 μM) was incubated for 15 minutes on the basolateral side of SCBN cells grown on Transwell supports. Following pretreatment, the cells were stimulated for 2 minutes and immunofluorescence analysis was performed as described above. In the presence of PAPA-NONOate, treatment with forskolin caused a 6-fold smaller change in the apical to basolateral CFTR immunofluorescence ratio compared to SCBN monolayers not treated with the NO donor (Figure 3.4).

It has long been known that one of the primary mechanisms whereby exposure to NO is transduced to a cellular response is through the stimulation of soluble guanylate cyclase activity (Rapoport et al., 1983). Pretreatment of SCBN monolayers with the guanylate cyclase inhibitor, 1H-(1,2,4)oxadiazolo-(4,3-a)quinoxalin-1-one (ODQ; 30 μM) (Garthwaite et al., 1995) failed to prevent the PAPA-NONOate-mediated reduction in responsiveness to forskolin (Figure 3.4). However, ODQ alone prior to forskolin stimulation also caused a reduction in the CFTR fluorescence ratio although not as great as ODQ and PAPA-NONOate together.

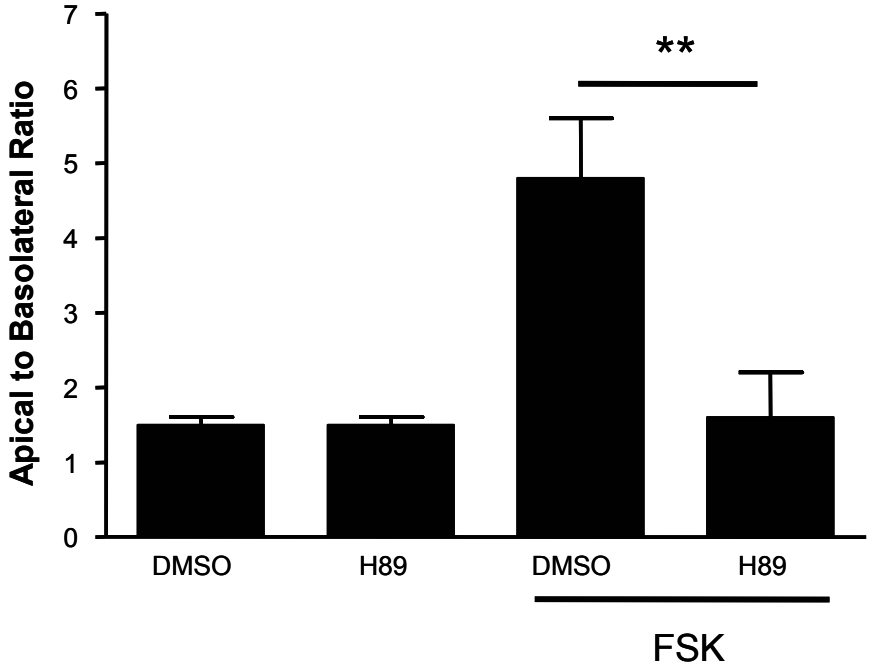


Figure 3.3. Effect of pretreatment of SCBN monolayers with the PKA inhibitor, H89 (5 μM) prior to basolateral exposure to forskolin (10 μM) for 2 min, and subsequent fixation and staining for CFTR immunoreactivity. The distribution of CFTR was calculated as the ratio between apical and basolateral fluorescence. **p<0.01 between groups, n=5-7.

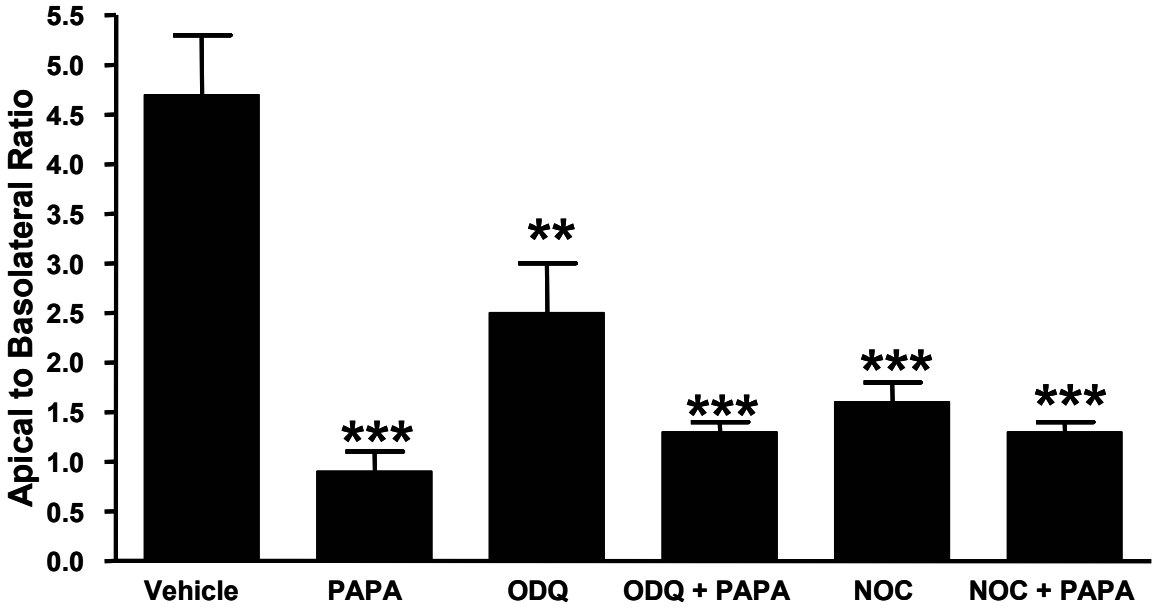


Figure 3.4. Effect of pretreatment of SCBN monolayers with the NO donor, PAPA-NONOate (100 μM) prior to exposure to forskolin (10 μM) or vehicle for 2 min, and subsequent fixation and staining for CFTR immunoreactivity. The distribution of CFTR was calculated as the ratio between apical and basolateral fluorescence. Exposure to PAPA-NONOate blocked the forskolin-induced increase in apical to basolateral ratio (control). This inhibition was not affected when cells were treated with the guanylate cyclase inhibitor, ODQ or the microtubule inhibitor nocodazole (NOC), prior to exposure to PAPA-NONOate and forskolin. ODQ or NOC treatment prior to exposure to forskolin also did not affect inhibition. Treatment with any of the compounds without forskolin stimulation did not show an increase in the ratio of apical to basolateral CFTR immunofluorescence (not shown). ** $p<0.01$, ***$p<0.001$ vs. vehicle.

Membrane trafficking of CFTR is microtubule-dependent and can be disrupted with nocodazole (Schwiebert et al., 1994). Pretreatment of SCBN monolayers with 33 μM nocodazole and/or 100 μM PAPA-NONOate also prevented the increase in the ratio of apical to basolateral CFTR immunofluorescence following forskolin stimulation (Figure 3.4).

To determine if there was an interaction among forskolin-stimulated CFTR trafficking, NO and microtubules, in terms of chloride secretion, SCBN cells were the grown on Snapwell filters and mounted in modified Ussing chambers. The cells were pretreated with 100 μM PAPA-NONOate, 33 μM nocodazole or both. The cells were stimulated with 10 μM forskolin and the change in short circuit current was observed. The forskolin-induced ΔIsc was reduced 32% following pretreatment with nocodazole and 36% following pretreatment with PAPA-NONOate ($p<0.05$ for both compared to vehicle controls). Following pretreatment with NOC and PAPA-NONOate, the ΔIsc was reduced by 36% ($p<0.05$ compared to vehicle control) (Figure 3.5).

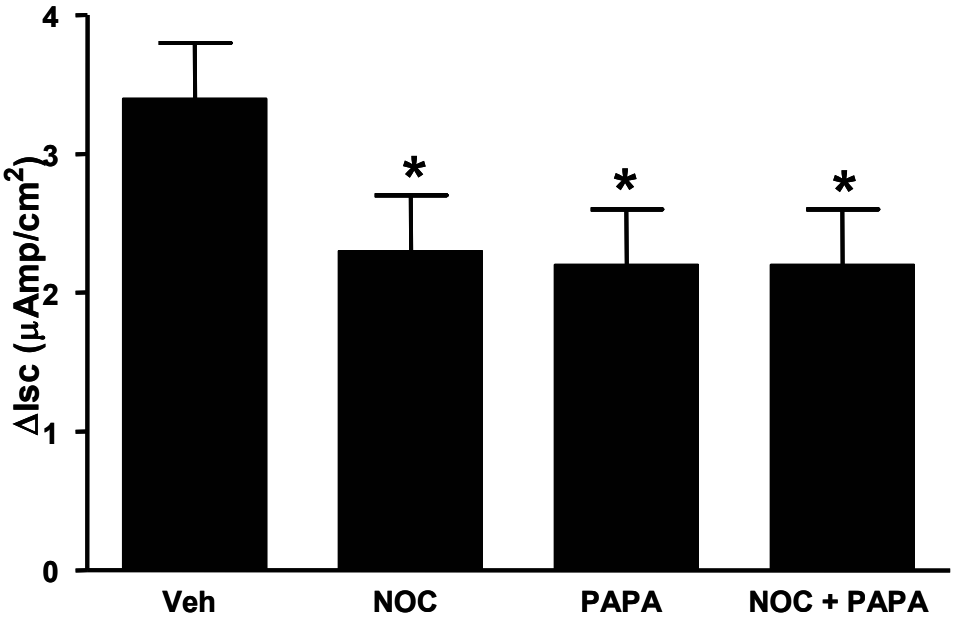


Figure 3.5. Short circuit current change (ΔIsc) in SCBN monolayers exposed to forskolin (10 μM). Monolayers were pretreated with vehicle (N=12), nocodazole (33 μM, N=12), PAPA-NONOate (100 μM, N=10) or a combination of the two (N=14) prior to exposure to forskolin. *p<0.05 vs. vehicle.

We also wanted to investigate a possible mechanism for the PAPA-NONOate-dependent reduction in forskolin induced ΔIsc. We measured cellular cAMP concentration following exposure to 100 μM PAPA-NONOate and/or 10 μM forskolin. The concentration of cAMP was significantly increased following forskolin alone, but not PAPA-NONOate alone. The combination of PAPA-NONOate and forskolin showed an increase in the concentration of cAMP. However, it was also significantly lower than forskolin alone showing that PAPA-NONOate partially reduced the concentration of cAMP following forskolin stimulation (Figure 3.6).

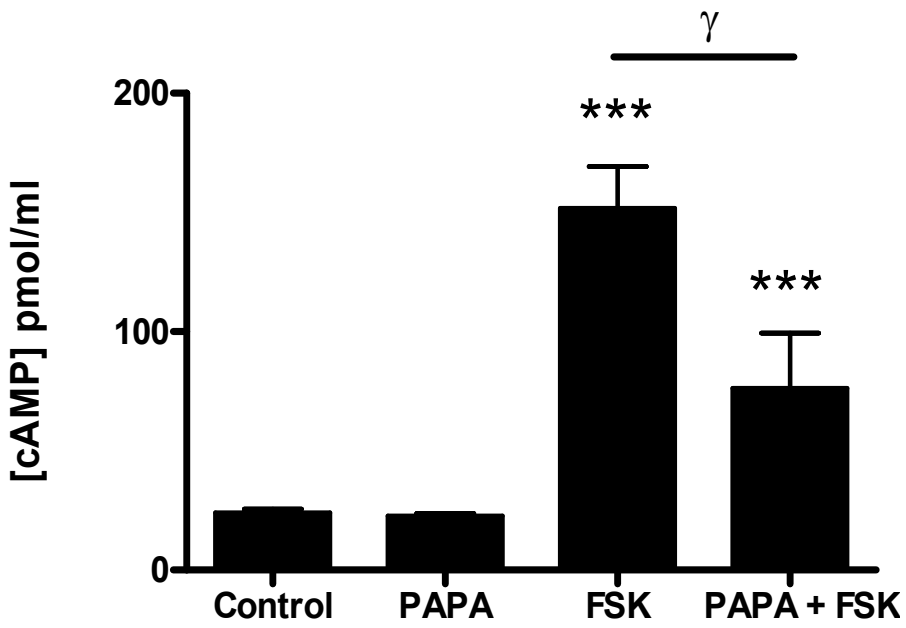


Figure 3.6. Cellular cAMP concentration in SCBN cells. Cells were pretreated with PAPA-NONOate (10 μM) and cAMP levels were determined in the presence and absence of forskolin (10 μM). *** $p<0.001$ vs. vehicle control. γ $p<0.05$ between groups. n=4.

3.4 Discussion:

In this study we have shown that a nitric oxide donor blocks the cytoplasm to plasma membrane trafficking of CFTR induced by forskolin in the SCBN cell line. This cell line was originally shown to be capable of vectorial, cAMP-dependent chloride secretion (Pang et al., 1996). We showed previously that it expressed the mRNA for CFTR (Buresi et al., 2001) and now show that it expresses CFTR in cytoplasmic and plasma membrane pools. In addition, we have shown that forskolin-induced trafficking of CFTR is PKA-dependent and that it is inhibited by a nitric oxide donor. The inhibitory effect of the NO donor is not due to stimulation of guanylate cyclase.

The cycling of CFTR from cytoplasmic pools to the apical plasma membrane and back is a complex series of events dependent upon the cytoplasmic level of cAMP (reviewed in Bertrand and Frizzell, 2003). The insertion of CFTR into the apical plasma membrane has been observed after stimulation of adenylate cyclase in cell lines (Schwiebert et al., 1994; Tousson et al., 1996) and, more recently, in rat jejunum (Ameen et al., 2003). CFTR cycling begins with synthesis of the protein in the endoplasmic reticulum, with trafficking through the Golgi apparatus and into vesicles destined for insertion into the apical plasma membrane or an early/recycling endosome. The recycling endosome is both a source of CFTR to be inserted into the membrane by exocytosis or a sink of CFTR removed from the membrane by endocytosis (Bertrand and Frizzell, 2003). The early endosome is also a source of CFTR to be targeted to late endosomes for lysosomic degradation (Bertrand and Frizzell,

2003). Both the exocytosis and endocytosis events are controlled by intracellular levels of cAMP and activation of PKA (Bradbury, 2001). In addition, the channel activity of CFTR present in the membrane is a highly coordinated phosphorylation event regulated by cAMP and PKA (Gadsby and Nairn, 1999).

In the present study, we showed that exposure to 10 μM forskolin on the basolateral side of SCBN monolayers mounted on Snapwells caused a movement of CFTR immunoreactivity from the cytoplasm to the apical plasma membrane. We have previously shown that this dose of forskolin significantly increases cAMP in SCBN cells (Buresi et al., 2001) and have verified that in this study. To show the intracellular translocation of CFTR, we used an adaptation of the method of Moyer and Stanton (Moyer and Stanton, 2002). The apical plasma membrane was demarcated in cross sections of SCBN monolayers using staining for the tight junctional protein, ZO-1. CFTR staining was calculated as the ratio of apical to basolateral staining, where "basolateral" refers to the fluorescence intensity in the basolateral membrane minus the fluorescence intensity of the nuclear compartment (background).

We have investigated the relationship between forskolin-induced CFTR trafficking to the plasma membrane and increases in Isc. It was not possible to make this comparison directly, since trafficking was determined immunocytochemically in fixed cells. Interestingly, the period of peak insertion of CFTR into the plasma membrane following exposure to forskolin preceded the peak change in Isc elicited by forskolin. This is what would be expected if insertion of membrane played a part in the secretory response to forskolin, as

opposed to the response being due only to phosphorylation and gating of CFTR already in the plasma membrane. Indeed, in earlier studies it was shown that increasing cAMP in airway epithelial cells resulted in concomitant insertion of CFTR into the plasma membrane and chloride conductance as determined by patch clamp (Schwiebert et al., 1994). In our study, the peak apical:basolateral ratio of CFTR occurred at 30 s following exposure to forskolin, whereas the forskolin-induced change in Isc occurred at 2 min. This observation, together with the fact that disrupting microtubules with nocodazole also reduced forskolin-induced Isc (discussed below), suggests that CFTR trafficking plays a role in the rapid secretory response to activation of adenylate cyclase.

We also observed that forskolin-induced CFTR trafficking into the apical plasma membrane was a transient event. The apical:basolateral ratio of CFTR immunofluorescence was not significantly above baseline at 4 or 10 min after exposure to forskolin. This observation is consistent with previous reports showing that endocytosis of CFTR, effectively removing CFTR from the apical plasma membrane, is also a rapid event. Internalization of CFTR occurs within about 1 min in T84 cells (Prince et al., 1994), and while this is inhibited by forskolin, there is no difference in apical membrane CFTR by approximately 8 minutes after exposure to forskolin (Prince et al., 1994). This also raises the point that the observations we have made are likely a combination of increased insertion into the membrane and decreased endocytosis of CFTR out of the apical plasma membrane.

In the present study, we showed that the forskolin-induced trafficking was dependent upon PKA, since the effect was blocked by pretreatment of the cells with the PKA inhibitor, H89. The PKA isotype involved was not delineated by our studies, although previous reports would suggest that it is type II PKA that is involved, since analogues of this isotype are known to stimulate CFTR (Bradbury, 2001).

One of the novel and important findings of the present study is the observation that a NO donor blocked forskolin-induced CFTR trafficking. It has long been known that endogenous NO can promote fluid absorption in *in vivo* models. In rat small intestine, inhibition of NOS with L-NAME enhanced basal absorption (Schirgi-Degen and Beubler, 1995) and inhibited the secretion caused by PGE_2, cholera toxin, 5-HT or heat stable enterotoxin (Beubler et al., 1993; Beubler and Schirgi-Degen, 1997; Schirgi-Degen and Beubler, 1995). Increased iNOS expression is characteristic of inflammatory bowel disease (Singer et al., 1996; Guslandi, 1998), where the secretory function of the epithelium is impaired (Archampong et al., 1972; Sandle et al., 1990; Hubel and Renquist, 1990). Indeed, we have shown that iNOS-derived NO mediates inflammation-induced suppression of responses to cAMP-dependent secretagogues in TNBS colitis (MacNaughton et al., 1998) and radiation enteropathy in mice (Freeman and MacNaughton, 2000). In a rat model of mucosal recovery from inflammation, the response to cAMP-dependent secretagogues was still suppressed 6 weeks after a bout of inflammation, a time when iNOS expression was still elevated (Asfaha et al., 1999). Interestingly, secretory function as measured *in vitro* returned to

normal when tissues were treated with an iNOS inhibitor (Asfaha et al., 1999). Of significance is the fact that inhibition of iNOS and return to normal secretory function in the post-inflammatory bowel also prevented the increased bacterial translocation observed in this model (Asfaha et al., 2001). Recently we showed the effect of NO was due to inhibition of NO-sensitive isoforms of adenylate cyclase (Freeman and MacNaughton, 2004). What remained unknown was whether NO inhibited cAMP-dependent CFTR trafficking, gating or both.

In the present study we have shown that the NO donor PAPA-NONOate reduced the forskolin-induced increase in apical CFTR. Previously we showed that PAPA-NONOate and another NO donor, sodium nitroprusside, reduced forskolin-induced accumulation of cAMP in both mouse colon and T84 colonic epithelial cells, and that both of these NO donors inhibited changes in Isc induced by forskolin in segments of mouse colon mounted in Ussing chambers (Freeman and MacNaughton, 2004). Since many of the cellular effects of NO are mediated through activation of guanylate cyclase and increased cytoplasmic cGMP (Rapoport et al., 1983), we repeated the studies of PAPA-NONOate-induced blockade of forskolin-induced CFTR trafficking in cells pretreated with the guanylate cyclase inhibitor, ODQ (Garthwaite et al., 1995). Interestingly, ODQ pretreatment failed to block the PAPA-NONOate effect, suggesting that NO was not acting through guanylate cyclase. This is in agreement with our previous work that showed that NO donors failed to increase cGMP in T84 cells or mouse colon (Freeman and MacNaughton, 2004). However, ODQ alone followed by

forskolin also had an effect in reducing the ratio of apical to basolateral CFTR immunofluorescence, although not as great as the PAPA-NONOate effect.

Microtubules have been shown to mediate the cytoplasm to apical plasma membrane trafficking of CFTR stimulated by increases in cAMP (Tousson et al., 1996). This study and others (Schwiebert et al., 1994) used the microtubule inhibitor, nocodazole, to demonstrate cAMP dependent trafficking of CFTR-containing membrane vesicles. In order to determine if the effect of the NO donor was on microtubules, we pretreated SCBN monolayers mounted in Ussing chambers with PAPA-NONOate or nocodazole or both, prior to challenge with forskolin. At the concentrations used, both nocodazole and PAPA-NONOate significantly reduced the forskolin-induced increase in Isc (by 32 and 36% respectively). Interestingly, the reduction of forskolin-induced Isc by the combination of PAPA-NONOate and nocodazole was approximately the same (36%). This observation suggests that PAPA-NONOate and nocodazole are acting at the same pathway, or at points in series along the same pathway, rather than on distinct, parallel pathways. If the two compounds were acting on separate pathways, one would expect the combination would have an additive inhibitory effect on forskolin-induced Isc. However, this was not the case. We also observed a similar reduction in the ratio of apical to basolateral CFTR immunofluorescence following pretreatment with nocodazole and/or PAPA-NONOate and exposure to forskolin. This also reinforces the concept that nocodazole and PAPA-NONOate are acting at the same pathway. Based on our data and the techniques used to obtain them, and given the fact that cAMP-

dependent CFTR trafficking is microtubule-dependent, several interpretations are possible. First, PAPA-NONOate could be acting to inhibit adenylate cyclase, thus reducing the forskolin-induced increase in intracellular cAMP that stimulates CFTR trafficking. If cAMP is not increased, then trafficking is not stimulated and any effect of nocodazole on microtubules would be irrelevant in terms of Isc. We show in this study that cAMP is significantly increased following forskolin and combined treatment of forskolin and PAPA-NONOate. However, there is a significant reduction in the forskolin and PAPA-NONOate treatment versus forskolin alone. This reduction could therefore at least partially prevent some CFTR trafficking from taking place. Second, it could be that PAPA-NONOate is acting either directly or indirectly to disrupt microtubules itself, in which case forskolin-induced CFTR trafficking would be reduced, regardless of the presence or absence of nocodazole. Additional studies will be necessary to determine which of these mechanisms, or if another mechanism, is more responsible and relevant for the observations. In either case, it is clear that CFTR trafficking is responsible for a significant proportion of the change in Isc elicited by exposure to forskolin, and that this is sensitive to NO.

In summary, we have shown that cAMP-dependent trafficking of CFTR from cytoplasmic stores to the apical plasma membrane can be rapidly induced by increasing cAMP in SCBN cells. This process can be blocked by NO in a guanylate cyclase-independent fashion. The ability of NO to disrupt the action of cAMP-dependent secretagogues has implications in terms of the ability of the

intestinal epithelium to maintain the secretory component of its barrier function in conditions when mucosal iNOS is elevated.

Chapter 4: Effect of Nitric Oxide on Tight Junctions and Permeability in Epithelial Cells:

4.1 Introduction:

Having assessed the effect of NO on CFTR trafficking and function in the epithelium, we next studied the role of NO in another component of epithelial host defense function, namely the tight junction. Tight junctions are formed by cell-cell adhesions around the cell and control paracellular permeability of fluid and solutes (Yap et al., 1998). The tight junctional complex is comprised of both transmembrane proteins and cytosolic proteins that are recruited to the membrane. Transmembrane proteins include occludin and members of the claudin family. An example of a cytosolic protein recruited to the complex is zonula occludens protein 1 (ZO-1). ZO-1 can regulate paracellular permeability since it interacts with occludin at its N terminus and with cytoskeletal F-actin at its C terminus (Anderson and Van Itallie, 1995).

Disruption of proteins within the tight junctional complex can lead to increased permeability in the epithelial layer (Mitic and Anderson, 1998) and may provide a route whereby pathogens such as bacteria may cross the epithelium (Neish, 2002). The epithelium has a number of defense mechanisms to prevent bacterial translocation. Occasionally, as in situations of host injury, inflammation, or infection, bacteria are able to evade these defenses. However, the precise mechanism involved in bacteria translocating through the intestinal epithelium is still not clear.

Since nitric oxide has been shown to be involved in the host response to bacterial infection, it is also possible that it contributes to bacterial translocation and/or changes in epithelial permeability. In studies using human intestinal epithelial cell lines, transepithelial resistance was decreased following exposure to enteroinvasive bacterial species, *E. coli* 029:NM or *Salmonella dublin*, and this decrease was blocked by iNOS inhibitors (Resta-Lenert and Barrett, 2002). Furthermore, following exposure to cytokines such as IFN-γ, NO produced by iNOS has been shown to alter tight junction protein organization and expression (Fink and Delude, 2005).

In this study, SCBN and CMT-93 epithelial cells were assessed for disruption of tight junctional proteins as well as changes in permeability following exposure to a nitric oxide donor. We demonstrate that the changes in chloride secretion and CFTR translocation found after addition of a nitric oxide donor shown in the previous chapter do not occur due to changes in epithelial permeability.

4.2 Materials and Methods:

4.2.1 Cell Lines:

SCBN cells were grown as described in Chapter 2.1.1.

CMT-93 is a mouse tumorigenic cell line isolated from the large intestine (Franks and Hemmings, 1978). The cells have epithelial morphology and tight junctional complexes. Cells (passages 45-47; obtained from Dr. A. Buret, University of Calgary) were grown to confluence in culture flasks and seeded on Snapwell membranes. Cells were fed Dulbecco's Modified Eagle Medium

(DMEM) supplemented with 5% fetal bovine serum (FBS), L-glutamine, streptomycin and tylosin. An electrovoltohmmeter (EVOM, World Precision Instruments, Sarasota, FL) was used to measure resistance of the cells and only cells with a resistance of 1000 Ohms/cm^2 or greater were used for experiments.

4.2.2 Immunofluorescent staining of tight junctions:

SCBN monolayers were grown on chamber slides as described above and stained according to protocols described in 2.2. Table 4.1 indicates the antibodies and concentrations used. Images were taken as described in Chapter 1.2 using a 40X objective.

4.2.3 FITC-Dextran 3000 Permeability Assay:

Cells were assessed for permeability using the FITC-Dextran 3000 MW assay as previously described (Chin et al., 2002). Briefly, cells were grown on Transwell filters as described above to a resistance of greater than 1000 Ohms/cm^2. Cells were pretreated with 100 μM PAPA-NONOate, vehicle or were not treated. FITC-Dextran MW 3000 (Molecular Probes, Eugene, OR) was added to the apical side of each monolayer to a final concentration of 10 μM in bicarbonate buffered Ringer's Solution (115 mM NaCl, 50 mM $NaHCO_3$, 2.8 mM KH_2PO_4, 2.8 mM K_2HPO_4, 1.2 mM $CaCl_2$, 1.2 M $MgCl_2$, and 10 mM D-Glucose). Monolayers were incubated for three hours at 37°C. 300 μL samples were taken from the basolateral compartment and read in duplicate on a microplate fluorometer (Excitation 496 nm, Emission 524 nm, cutoff 530 nm; Spectra Max Gamini, Molecular Devices, Sunnyvale, CA). The readings were then expressed

Primary Antibodies	Supplier	Dilution
Rabbit Anti-ZO-1	Zymed	1:100
Mouse Anti-claudin-4	Zymed	1:250
Mouse Anti-claudin-5	Zymed	1:250
Mouse Anti-occludin	Zymed	1:250

Secondary Antibodies	Supplier	Dilution
Goat-anti-rabbit-FITC	Molecular Probes	1:200
Goat-anti-mouse-Cy3	Jackson	1:1000

Table 4.1. Antibodies and the dilutions used for immunofluorescent staining of tight junctional proteins. The antibodies in the table were used at the indicated concentrations according to the protocols outlined in Chapter 2.2.

as a percentage of the initial fluorescence of the FITC-Dextran 3000 MW solution added to the apical side of the monolayers.

4.2.4 Bacterial Translocation Assay:

Cells were grown on Snapwell filters as described above to a resistance of at least 1000 Ohms/cm^2. 24 hours before exposing the monolayers to bacteria, the medium was replaced in both apical and basolateral compartments with antibiotic-free media.

C. rodentium, strain DBS 100, a generous gift from Dr. David Schauer (MIT, Cambridge, MA), were grown under aerophilic conditions on MacConkey agar plates overnight at 37^0C. Bacteria were then harvested and grown in Penassay broth overnight at 37^0C. On the day of the experiments, cell monolayers were infected with 10^8 live bacteria and incubated for 2 hours at 37^0C. 100 μl samples were taken from the apical and basolateral compartments, serial diluted and plated onto MacConkey agar plates. The plates were incubated overnight at 37^0C. For the apical compartment samples, plating was performed to ensure bacteria remained viable throughout the experiment. For the basolateral compartment, colony forming units (CFU) were counted and expressed as CFU/ml based on the dilution factors.

4.3 Results:

4.3.1 Effect of Nitric Oxide on Tight Junction Proteins:

SCBN cells were stained using immunofluorescence for the tight junctional proteins claudin-4, claudin-5, occludin and ZO-1. For each protein, vehicle treatment resulted in fluorescence at the junctions between each cell. Viewing the surface of the monolayer, tight junctional proteins appear to outline the cells in a honeycomb pattern. Upon treatment with 100 μM PAPA-NONOate, there was no change in the staining for claudin-4, claudin-5 and occludin, as the honeycomb pattern was still present and intact (Figures 4.1-4.3). However, in the case of ZO-1, the honeycomb fluorescent pattern became disrupted following treatment with the NO donor at 100 μM (Figure 4.4). At 10 μM PAPA-NONOate, however, the ZO-1 immunofluorescence did not differ from vehicle control.

4.3.2 Effect of Nitric Oxide on Epithelial Permeability:

Epithelial permeability was assessed using two different assays. In the first experiment, the FITC-Dextran 3000 MW permeability assay was used. SCBN cells grown on Transwell filters were treated with 100 μM PAPA-NONOate, vehicle, or were not treated. After three hours, there was no significant difference in the percentage of FITC-Dextran 3000 MW collected in the basolateral compartments for any of the groups being assayed. In fact, less than 1% of the total FITC-Dextran 3000 MW added to the apical compartment was recovered in the basolateral compartment in all groups (Table 4.2).

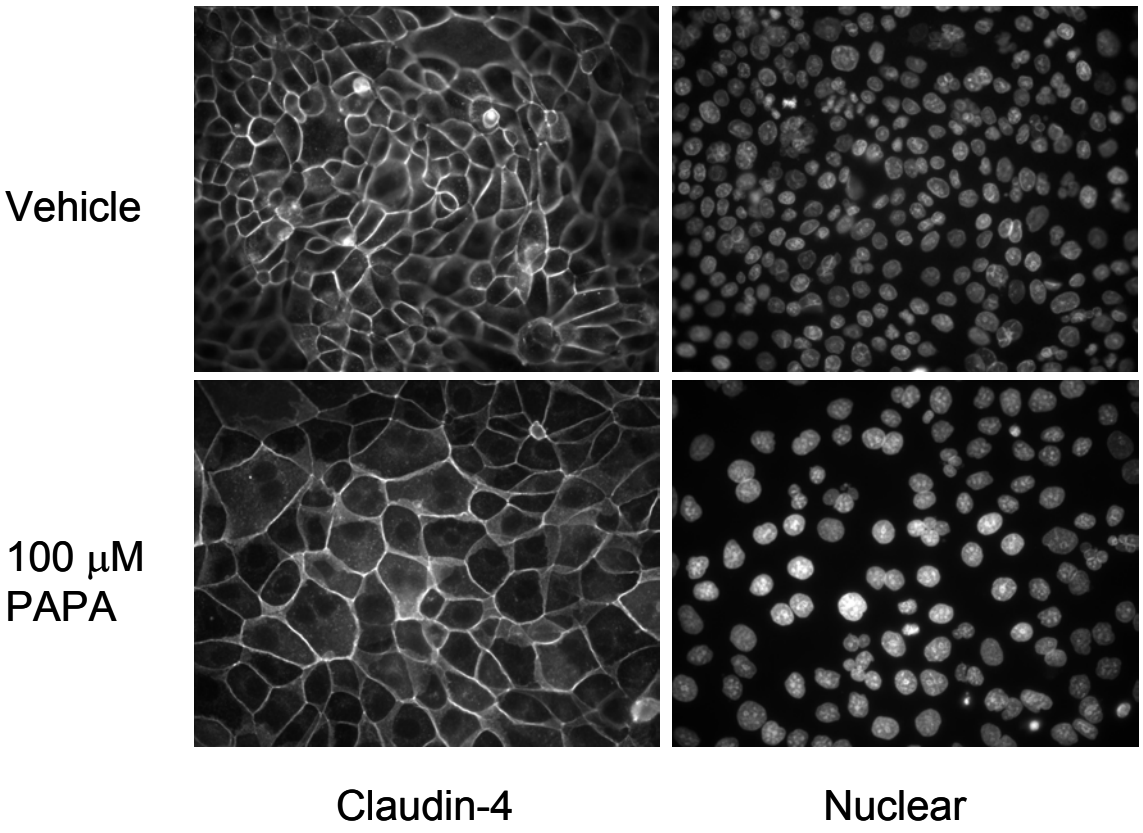


Figure 4.1. Claudin-4 immunofluorescence in SCBN cells following exposure to a NO donor. SCBN cells were treated for 20 minutes with the NO donor PAPA-NONOate (100 μM), fixed, and stained for tight junctional protein claudin-4 (left) and nuclear staining with bis-benzimide (right) (40X objective; N=3-4 for each treatment).

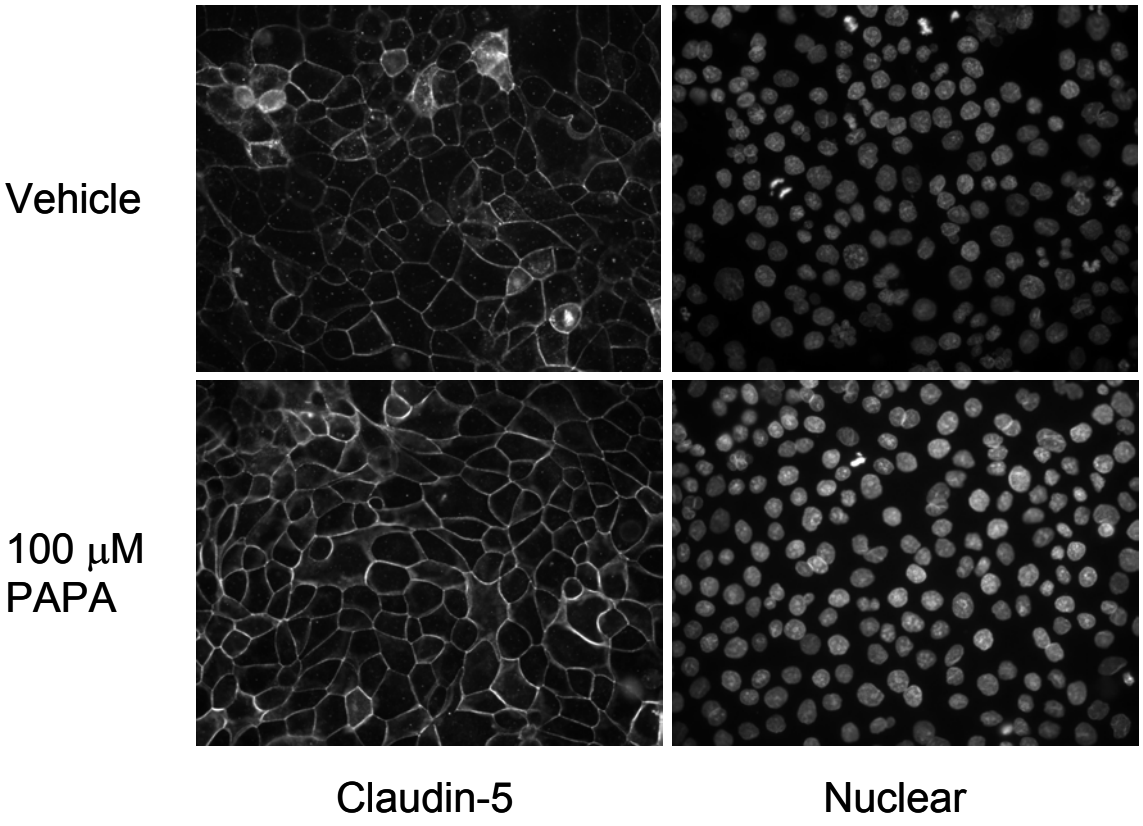


Figure 4.2. Claudin-5 immunofluorescence in SCBN cells following exposure to a NO donor. SCBN cells were treated for 20 minutes with the NO donor PAPA-NONOate (100 μM), fixed, and stained for tight junctional protein claudin-5 (left) and nuclear staining with bis-benzimide (right) (40X objective; N=3-4 for each treatment).

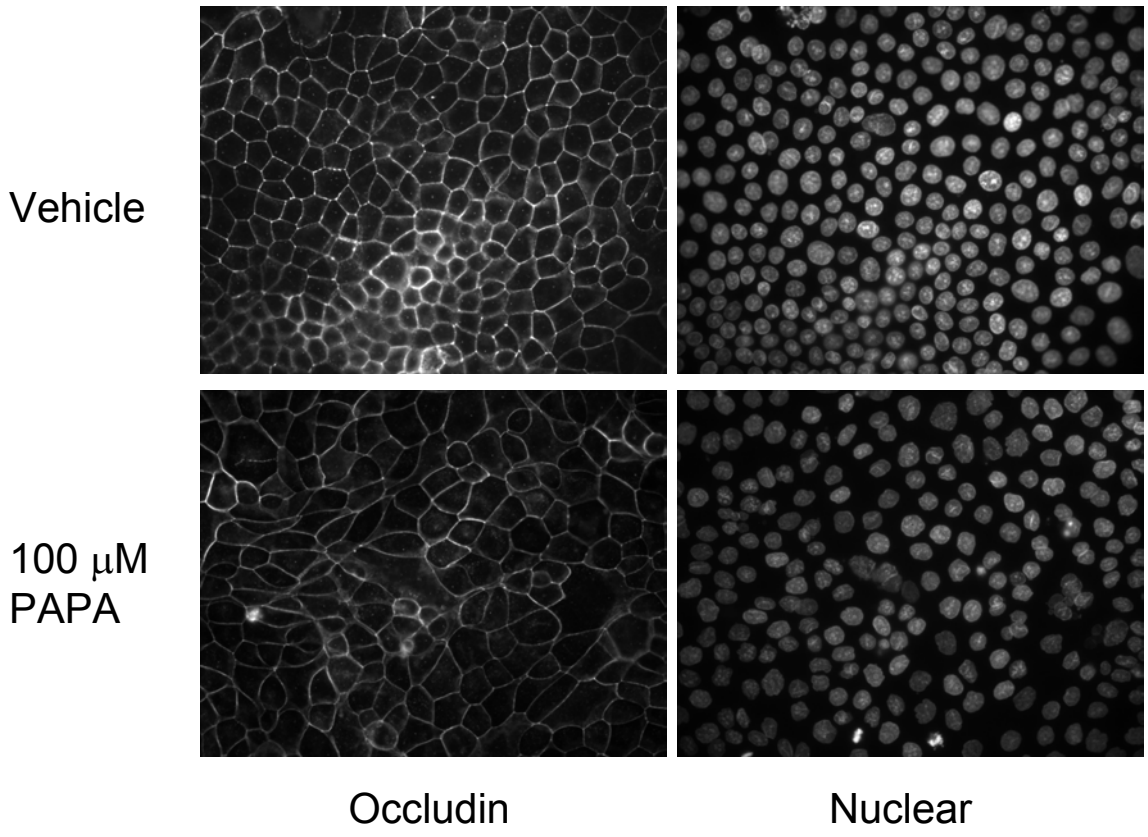


Figure 4.3. Occludin immunofluorescence in SCBN cells following exposure to an NO donor. SCBN cells were treated for 20 minutes with the NO donor PAPA-NONOate (100 μM), fixed, and stained for tight junctional protein occludin (left) and nuclear staining with bis-benzimide (right) (40X objective; N=3-4 for each treatment).

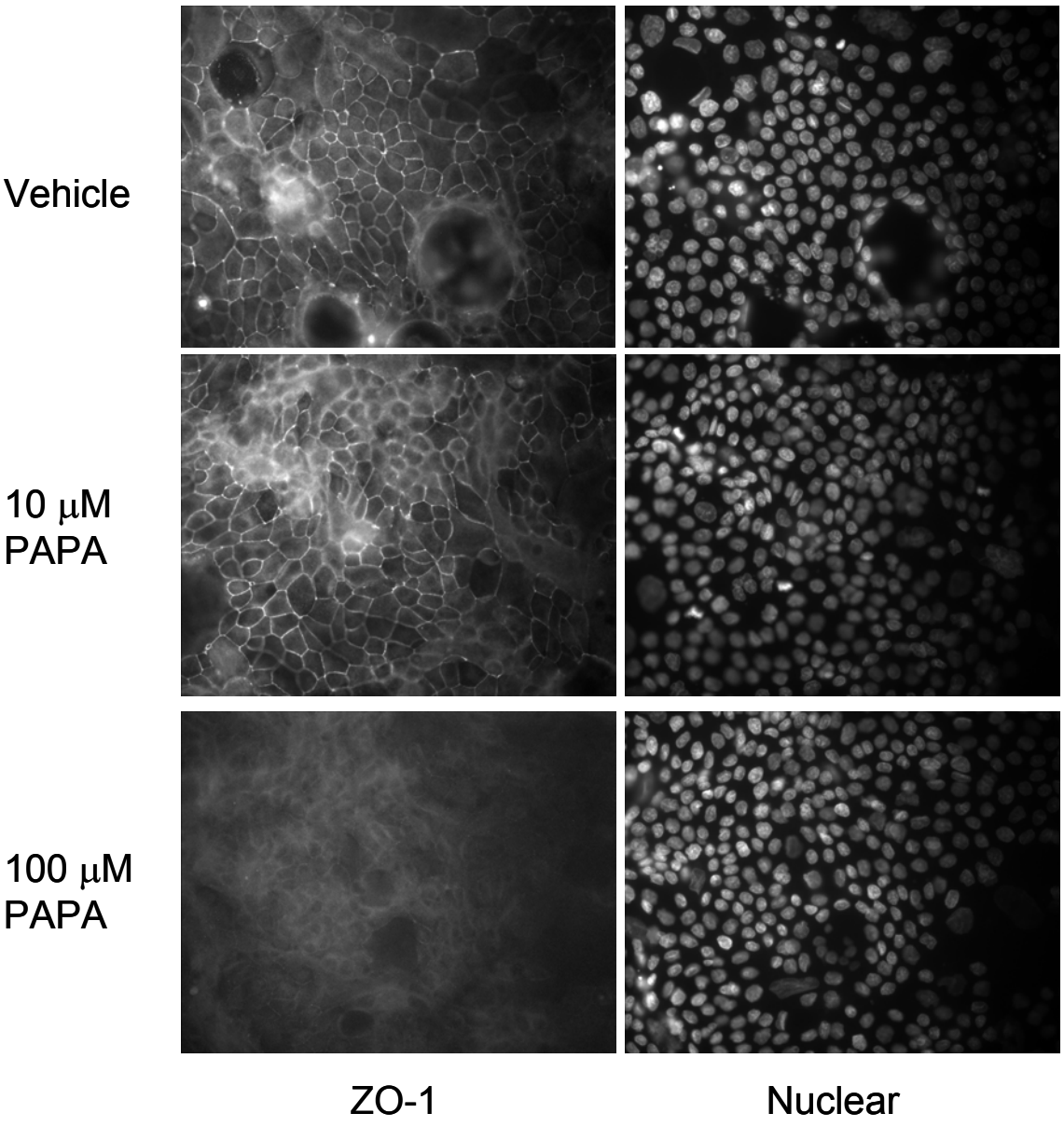


Figure 4.4. ZO-1 immunofluorescence in SCBN cells following exposure to a NO donor. SCBN cells were treated for 20 minutes with the NO donor PAPA-NONOate (10 and 100 μM), fixed, and stained for tight junctional protein ZO-1 (left) and nuclear staining with bis-benzimide (right) (40X objective; N=3-4 for each treatment).

Group	% apical FITC-Dextran /cm^2
No treatment	0.54 ± 0.03
NaOH vehicle	0.54 ± 0.06
100 μM PAPA-NONOate	0.58 ± 0.05

Table 4.2. FITC-Dextran 3000 MW epithelial permeability following treatment with a nitric oxide donor. Transepithelial fluxes of FITC-Dextran 3000 MW across SCBN epithelial monolayers pretreated with or without 100 μM PAPA-NONOate. Values are mean ± SEM; n = 4, p>0.05 between groups.

To verify the above results, we used another permeability assay using *Citrobacter rodentium* as the marker of permeability. SCBN cells were grown on Snapwell filters and treated with 100 μM PAPA-NONOate or vehicle. *C. rodentium* was added to the apical side and two hours later, media from the basolateral compartment was sampled and dilutions were plated. In both the monolayers treated with the NO donor and the vehicle treated group (n = 6), no *C. rodentium* was cultured from the basolateral samples. *C. rodentium* was cultured from the apical compartment indicating that live bacteria were still present at the end of the incubation period.

Since *C. rodentium* is a mouse pathogen, we decided that the above results should be verified using a mouse cell line to ensure that the reason for *C. rodentium* not crossing the monolayer was not due to species-specificity. CMT-93 cells were grown on Snapwell filters and treated with 100 μM PAPA-NONOate or vehicle. Following the same protocol as for the SCBN cells, samples were again collected from the basolateral compartment, diluted and plated. Again, in both the monolayers treated with the NO donor and the vehicle treated group (n = 6), no *C. rodentium* was cultured from the basolateral samples. *C. rodentium* was cultured from the apical compartment indicating that live bacteria were still present at the end of the incubation period.

4.4 Discussion:

This study shows that in SCBN epithelial cells, nitric oxide can disrupt tight junctional protein ZO-1, but not occludin, claudin-4 or claudin-5. However,

disruption of ZO-1 is not accompanied by an increase in permeability as measured by FITC-Dextran 3000 MW or *C. rodentium* translocation.

As discussed above (Chapter 1), eNOS and nNOS are the constitutive isoforms producing nitric oxide whereas iNOS is the inducible isoform. It is likely that the constitutive levels of nitric oxide present in the gut are responsible for regulating normal intestinal barrier function. Ileal permeability can be altered by non-specific inhibition of nitric oxide synthesis (Kubes, 1992). In other experiments of gut mucosal permeability using ischaemia-reperfusion and endotoxin challenge, a nitric oxide donor reversed the hyperpermeability that is usually observed in these models (Payne and Kubes, 1993). However, it has also been shown during acute ischaemia/reperfusion injury or LPS stimulation, that inhibition of nitric oxide synthases worsens intestinal barrier dysfunction (Alican and Kubes, 1996; Hutcheson et al., 1990). This suggests that at normal concentrations, nitric oxide is an important regulator in gut function whereas upon removal, it allows mucosal integrity to be compromised more easily.

Overproduction of nitric oxide, such as that following up-regulation of iNOS, presents a different situation for the gut from the normal constitutive levels. Evidence has shown that the epithelium may lose normal function when high concentrations of nitric oxide are present. For example, damage to the gastric mucosa of rats is observed following infusion of nitric oxide donors at high concentrations (Lopez-Belmonte et al., 1993). Similar results are seen in rat intestinal epithelial cells in that viability is decreased following treatment with nitric oxide donors (Tepperman et al., 1993). Epithelial cell monolayers exposed

to nitric oxide at high concentrations show dilatation of tight junctions, actin cytoskeleton disruption and ATP inhibition (Salzman et al., 1995; Forsythe et al., 2002). All of these events lead to increased intestinal permeability. During chronic phases of inflammation, inhibition of nitric oxide synthesis reduces intestinal injury (Boughton-Smith et al., 1993). In a recent study, nitric oxide was shown to have a direct effect on barrier function (Xu et al., 2002). In this study, both ileal membranes and IE-6 intestinal epithelial cells were exposed to a nitric oxide donor. The membranes showed signs of mucosal damage and decreased in resistance whereas the IE-6 cells showed increases in cell death. Exposure of the cell lines to endotoxin produced increases in iNOS mRNA expression, nitric oxide production and permeability.

Bacteria have also been shown to regulate permeability in epithelial cell lines. T84 intestinal epithelial cells infected with EPEC show increased permeability measured by mannitol flux (Spitz et al., 1995). The disruption of permeability has been shown to be dependent on EspF being secreted by the type III secretion system (McNamara et al., 2001). EPEC can phosphorylate the 20 kDa myosin light chain kinase in T84 cells inducing cytoskeletal contraction (Yuhan et al., 1997). This in turn leads to dephosphorylation and dissociation of occludin from intercellular tight junctions (Simonovic et al., 2000). Other pathogens such as the protozoan *Giardia lamblia,* have also been shown to interact with an epithelial monolayer and disrupt ZO-1 leading to increased epithelial permeability (Chin et al., 2002).

While the above findings from other studies point to a role for nitric oxide in regulating epithelial permeability, our studies showed that nitric oxide delivered by a nitric oxide donor did not have an effect on permeability. The concentration of PAPA-NONOate used in these studies was 100 μM which is the same concentration which caused changes in CFTR trafficking and a reduction in the change in short circuit current in response to forskolin (Chapter 3). Therefore, the changes reported in Chapter 3 are occurring independently of permeability changes.

An intriguing finding is that nitric oxide appears to be selectively disrupting ZO-1 in SCBN epithelial cells. The mechanism and pathophysiological relevance of this apparent selectivity requires additional study. It appears that disruption of ZO-1 alone is insufficient to cause a change in permeability that would permit bacterial translocation. The permeability is maintained by occludin and the claudins which are not affected by the NO donor. These proteins form the "zipper-like" interactions in the paracellular space and therefore it is not surprising that if they are unaltered, epithelial permeability would be maintained. Another explanation could be that a longer exposure to NO may be required for further tight junctional protein disruption. In this study, the NO donor has a half-life of 20 minutes, whereas an NO donor that releases NO over a longer time period may have a different effect. Alternatively, ZO-1 may be the first protein that is disrupted by the NO donor and over a longer time period, the initial disruption of ZO-1 may lead to disruption of other tight junction proteins and cause a detectable change in epithelial permeability.

Another possibility is that the methods we are using to measure changes in permeability are not detecting a change due to ZO-1 production. In both methods used in this study, the size of the molecule or bacterium is the limiting factor in being able to detect changes in permeability. Perhaps a smaller probe would have revealed an NO-induced change in permeability not detectable with a larger probe. Alternatively, transepithelial resistance (TER) measurements could be taken before and after exposure to the NO donor to see if any changes occur since TER is often used as an indicator of ionic permeability. In these studies, we chose to use the FITC-Dextran and *C. rodentium* as a measure of changes in permeability since we felt these were appropriate indicators of physiologically relevant changes to epithelial permeability.

Chapter 5: Inflammation and Epithelial Dysfunction following *Citrobacter rodentium* infection:

5.1 Introduction:

To this point, we had assessed the effect of exogenous NO on different aspects of epithelial barrier function in a cell line system. The next objective was to determine barrier function in an animal model. Our aims were two-fold. First, we wished to assess barrier function, specifically ion transport and permeability, in a model of intestinal bacterial infection associated with inflammation. Second, based on our observations with a NO donor, we wished to determine if there was a role for NO in altered host defense function in this model.

Bacterial pathogens that cause attaching-effacing (A/E) lesions of the intestinal epithelium are important causes of infectious diarrhea worldwide (Kaper, 1998). Clinically important examples include enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) O157:H7. These differ from enteroinvasive bacterial species like *Yersinia*, *Shigella*, *Salmonella*, and *Campylobacter* in that they are non-invasive, infecting the host by attaching to the apical surface of intestinal epithelial cells through well defined molecular interactions (Kaper, 1998). While the mechanisms of attachment of A/E pathogens to the epithelial cell apical membrane have been studied in detail, there is still ambiguity as to how attachment leads to tissue damage and disease. Addressing this deficiency in our knowledge is best done using an *in vivo* model system. Although EPEC and EHEC have recently been shown to infect mice

(Savkovic et al., 2005), characterization of A/E pathogens in mice have largely focused on the mouse specific pathogen, *Citrobacter rodentium*.

Citrobacter rodentium is the causative agent of transmissible murine colonic hyperplasia (TMCH), which occurs naturally in laboratory mice (Barthold et al., 1978; Schauer et al., 1995). *C. rodentium* is currently the only known species-specific A/E pathogen capable of reproducibly infecting mice (Luperchio et al., 2000). Although *C. rodentium*-infected mice do not develop overt diarrhea, accumulation of fluid does occur in the colonic lumen. In addition, the A/E lesions formed by *C. rodentium* in mice are indistinguishable from those formed by EPEC and EHEC (Schauer and Falkow, 1993b). These characteristics have prompted the use of murine infection with *C. rodentium* as a model to study the mechanisms and consequences of the host response to A/E pathogens (Vallance and Finlay, 2000). However, no studies have assessed the effect of *C. rodentium* on epithelial barrier function.

Mounting evidence indicates that the lumen to tissue transmigration of a luminal factor is involved in the development of inflammatory bowel disease in genetically susceptible individuals (Guarner and Malagelada, 2003). Changes in epithelial permeability are associated with increased disease activity in some patients (Miki et al., 1998), and epithelial secretory function, a component of the epithelial barrier, is impaired during inflammation in animal models (Asfaha et al., 1999; MacNaughton et al., 1998). Loss of responsiveness to secretagogues following a bout of colitis increases bacterial translocation in animal models (Asfaha et al., 2001). However, the relationship between infection with an enteric

pathogen and barrier dysfunction associated with inflammation has not been studied in an animal model of infection. We hypothesized that infection of mice with *C. rodentium* would cause colonic inflammation with concomitant hyporesponsiveness to secretagogues. Since we have previously observed that secretory dysfunction during or after a bout of inflammation is associated with increased expression of the inducible isoform of nitric oxide synthase (iNOS) (Asfaha et al., 1999; MacNaughton et al., 1998), we also assessed the effect of *C. rodentium* infection on epithelial barrier function in knockout mice deficient in iNOS. The data indicate that *C. rodentium* infection causes prolonged colonic hyperplasia, mucosal inflammation and epithelial barrier dysfunction, associated with increased bacterial translocation, but that these occur independently of the induction of iNOS.

5.2 Materials and Methods:

5.2.1 Animals:

Male C57Bl/6 mice (Charles River, Montreal, PQ) were used for most experiments unless otherwise stated as described in Chapter 2.1.2. In some experiments rofecoxib or the vehicle, carboxymethyl cellulose (100 μL), was orally administered daily at a does of 10 mg/kg/day, starting 3 days after inoculation with *C. rodentium*. The mice were killed on day 10.

5.2.2 Citrobacter rodentium Infection:

Mice were infected as described in Chapter 2.1.2. Macroscopic damage score, wall thickness and MPO activity were assessed as described in Chapter 2.4. Bacterial translocation was assessed as described in Chapter 2.5.

5.2.3 Ion Transport Experiments:

Ussing chamber experiments were performed as outlined in Chapter 2.3. All compounds were added to the serosal chamber bath.

5.2.4 Western Blotting:

Western blotting was performed for iNOS and COX-2 expression as described in Chapter 2.6. These experiments were performed 10, 30 and 60 days following *C. rodentium* infection.

5.3 Results:

5.3.1 Histological Damage and Inflammation:

Following *C. rodentium* infection, mice did not exhibit signs of weight loss or lethargy. Upon laparotomy, in *C.* rodentium infected animals, edema of the colon was observed at day 7 and 10 post-infection. However, there was typically no erythema. A significantly higher macroscopic damage score was observed at 7 and 10 days following *C. rodentium* infection (Figure 5.1 A). There was a significant increase in colonic wall thickness at 3, 7, 10, 30 and 60 days post-infection (Figure 5.1 B). Myeloperoxidase activity was significantly elevated at 3, 7 and 10 days post-infection and returned to sham-infected levels by day 30 (Figure 5.1 C).

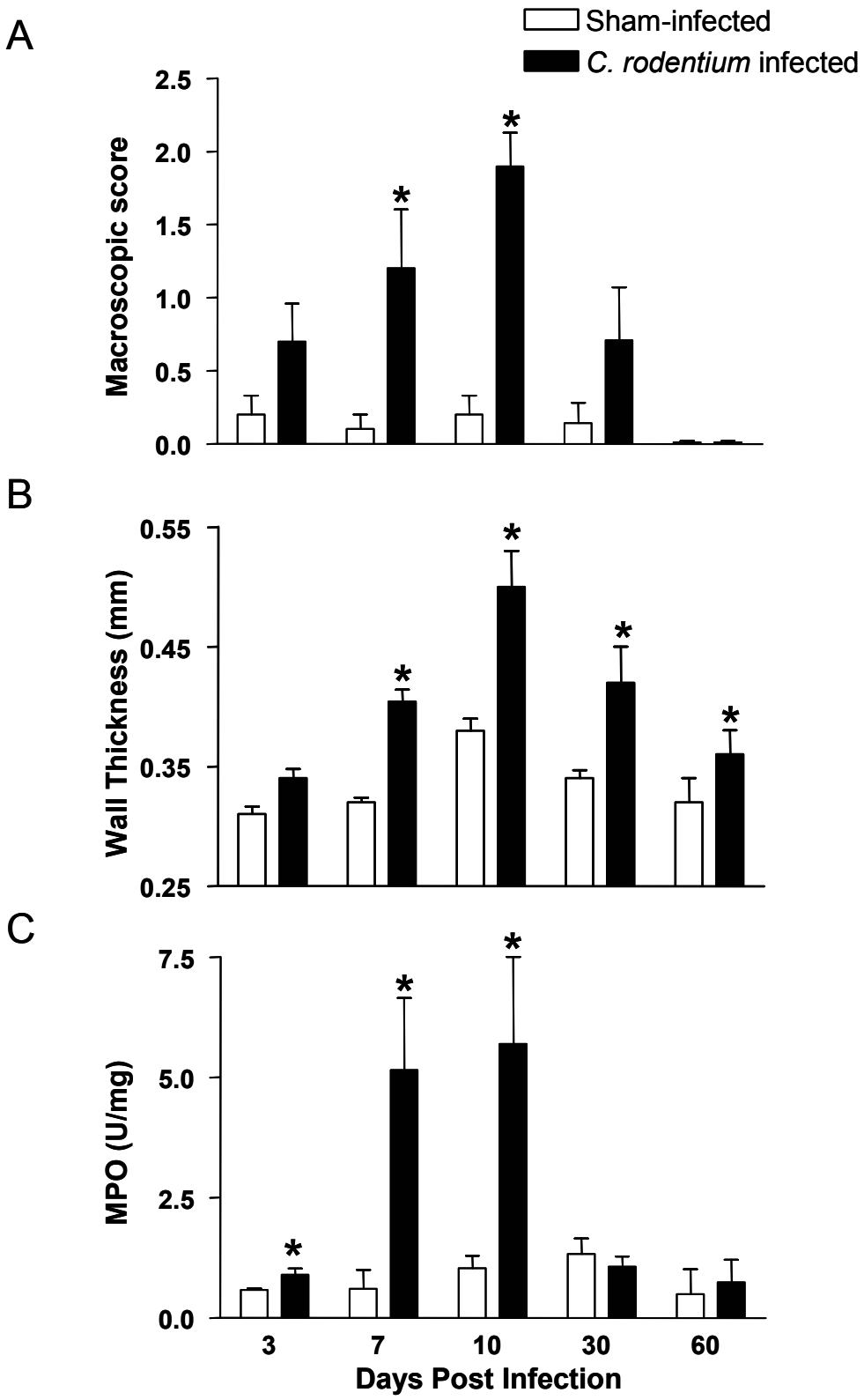

A
Sham-infected
C. rodentium infected
Macroscopic score
2.5
2.0
1.5
1.0
0.5
0.0
B
Wall Thickness (mm)
0.55
0.45
0.35
0.25
C
MPO (U/mg)
7.5
5.0
2.5
0.0
3
7
10
30
60
Days Post Infection

Figure 5.1.

Figure 5.1. Inflammatory response in mouse colon following *Citrobacter rodentium* infection. The following inflammatory parameters were measured at 3, 7, 10, 30 and 60 days following *Citrobacter rodentium* infection. A. Macroscopic damage scored according to the parameters in Chapter 2.4.1. B. Colon wall thickness measured using digital calipers. C. Myeloperoxidase activity in colonic tissue sections. * $p<0.05$ vs. sham-infected mice. N=10.

5.3.2 Ion Transport Experiments:

Ion transport experiments were performed to determine if the morphological changes caused by *C. rodentium* infection were associated with changes in net electrogenic ion flux. Conductance of the tissue was not significantly different between sham and infected groups at 3, 7, 10 or 30 days (Table 5.1). Electrical field stimulation, forskolin and carbachol caused immediate, transient increases in Isc in sham preparations. There was no statistical difference in the responses to EFS or forskolin between the sham and infected mouse colons (Figures 5.2 A and B). In contrast, addition of the cholinergic agonist carbachol (CCH), resulted in a significant reversal of current in infected colons at 3 and 10 days (Figure 5.2 C).

5.3.3 Bacterial Translocation:

Homogenates of mesenteric lymph nodes from sham-infected animals were negative for bacteria at all the time points investigated. In contrast, in *C. rodentium* infected animals, bacteria were cultured from mesenteric lymph nodes at 3, 7 and 10 days post-infection. The frequency of translocation was significantly different from sham-infected animals at day 10 following infection, with 8 of 22 animals showing the presence of bacteria (Table 5.2). At day 30, bacteria were no longer culturable from mesenteric lymph nodes in either group. No bacteria were present in homogenates of liver and spleen from any of the groups.

Days post-infection	Sham-infected (G)	*C. rodentium*-infected (G)
3	7.9 ± 0.7	7.5 ± 0.4
7	9.3 ± 0.6	8.4 ± 0.8
10	9.1 ± 0.5	8.4 ± 0.4
30	8.4 ± 0.7	8.9 ± 1.1

Table 5.1. Conductance of mouse colon following *Citrobacter rodentium* infection at 3, 7, 10 and 30 days post-infection. Values are expressed as mean ± SEM. N=6-14.

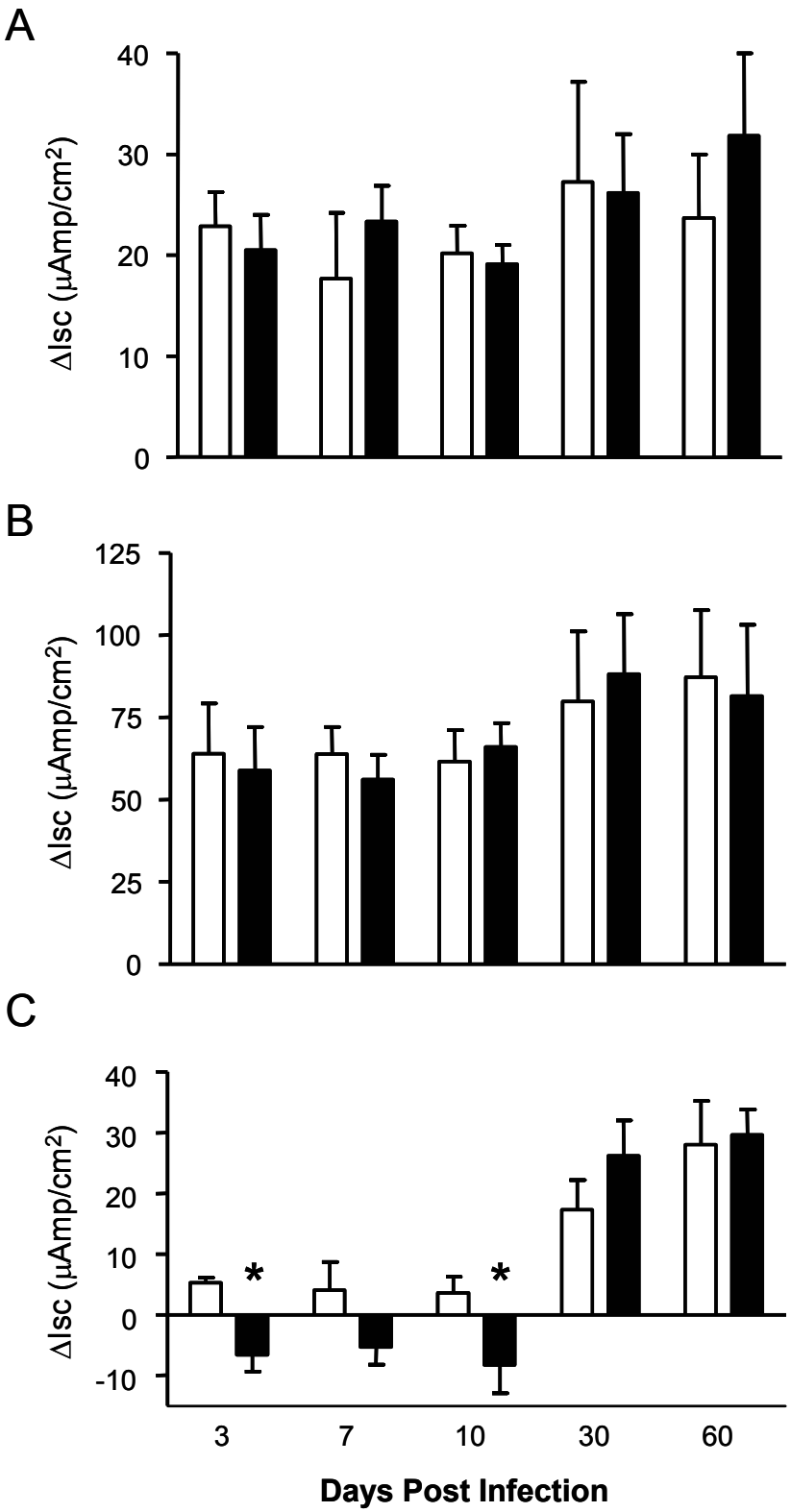

A
B
C
ΔIsc (μAmp/cm²)
40
30
20
10
0
125
100
75
50
25
-10
*
3
7
10
30
60
Days Post Infection

Figure 5.2.

Figure 5.2. Changes in short circuit current by secretagogues following *Citrobacter rodentium* infection. At 3, 7, 10, 30 and 60 days following *C. rodentium* infection, colons were removed and mounted in modified Ussing chambers and stimulated by electrical field stimulation (EFS; 50 V, 10 Hz, 5 s), forskolin (FSK; 10 μM) and carbachol (CCH; 5 μM). A. There was no significant difference in the change in short circuit current following EFS between infected and sham-infected animals. B. There was no difference in the change in short circuit current by forskolin stimulation between infected and sham-infected animals. C. At 3 and 10 days, *C.* rodentium infection caused a reversal in the change in short circuit current response to carbachol. * $p<0.05$ vs. sham-infected mice. N=6-14.

Days post infection	Sham	*C. rodentium*
3:	*0/10*	*3/10*
7:	*0/10*	*2/11*
10:	***0/21***	***8/22 ****
30:	*0/7*	*0/7*

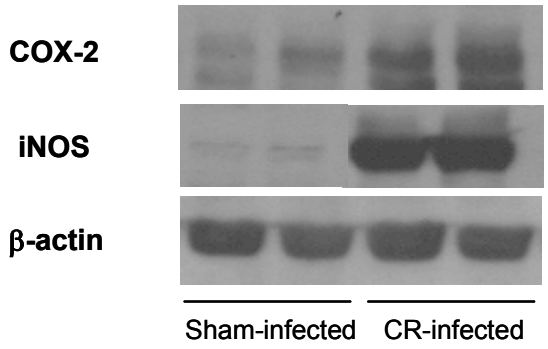
Table 5.2. Bacterial translocation in the mesenteric lymph nodes 10 days following *Citrobacter rodentium* infection. Bacterial translocation was identified as the presence of colonies following plating of the mesenteric lymph node homogenate on MacConkey agar. The ratios indicate the number of animals showing translocation over the total number of animals in the group. * $p<0.05$.

5.3.4 Role of iNOS and COX-2:

At 10 days post-infection, colonic expression of both iNOS and COX-2 was significantly increased compared to sham-infected mice, as determined by densitometry of immunoblots (Figure 5.3 A). At 30 (Figure 5.3 B) and 60 days (Figure 5.3 C) post-infection, COX-2 expression had returned to control levels. However, iNOS expression remained significantly elevated at both 30 and 60 days. As a result, we investigated the effects of *C. rodentium* infection in iNOS-deficient animals and the effects of the COX-2 inhibitor rofecoxib during infection.

There were no differences in *C. rodentium*-induced increases in macroscopic damage score (Figure 5.4 A), wall thickness (Figure 5.4 B) or MPO activity (Figure 5.4 C) in iNOS-deficient vs. wildtype controls at 10 days after *C. rodentium* infection. Similarly, iNOS-deficient mice showed no differences compared to wildtype in the change in short circuit current elicited by EFS or forskolin. The decreased response to carbachol observed in *C. rodentium*-infected mice was present in, and not significantly different between, iNOS-deficient and wildtype mice (Figure 5.5). Incidence of bacterial translocation to the lymph nodes was not different between *C. rodentium* infected wildtype (4 of 9) or iNOS-deficient mice (3 of 9).

A



B

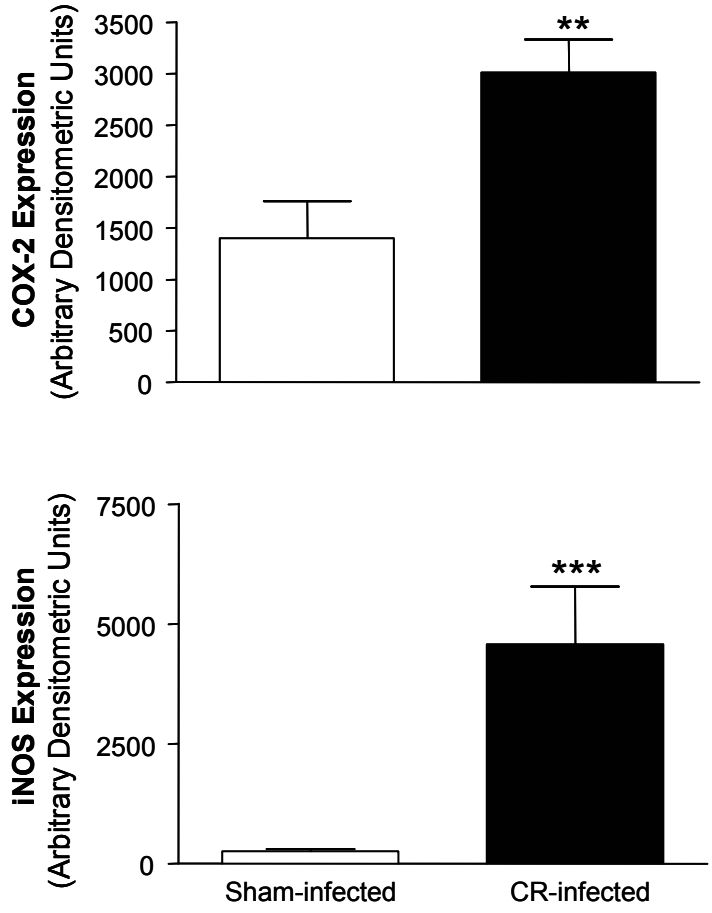


Figure 5.3 A.

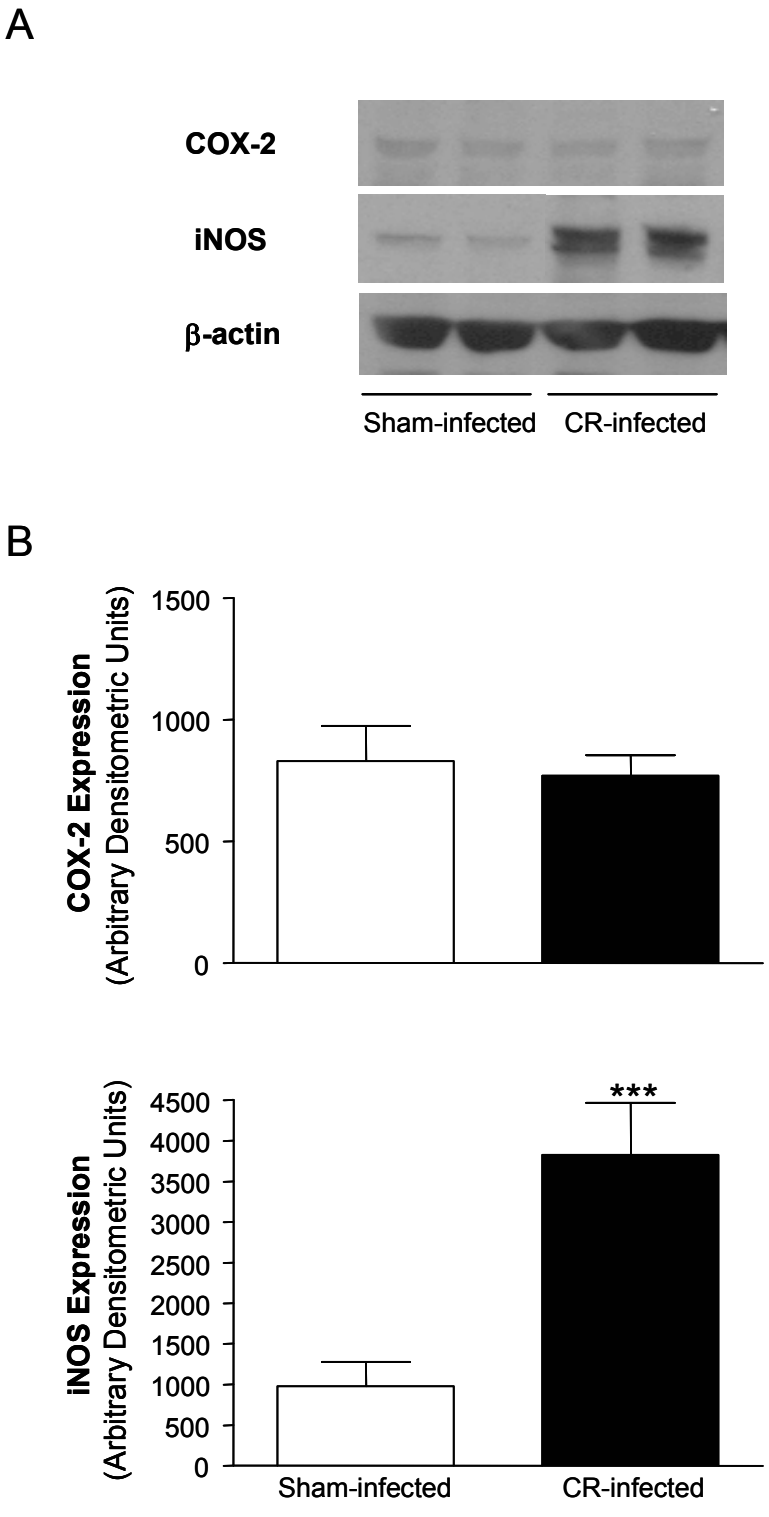

A
COX-2
iNOS
β-actin
Sham-infected
CR-infected
B
COX-2 Expression
(Arbitrary Densitometric Units)
1500
1000
500
0
iNOS Expression
(Arbitrary Densitometric Units)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
***
Sham-infected
CR-infected

Figure 5.3 B.

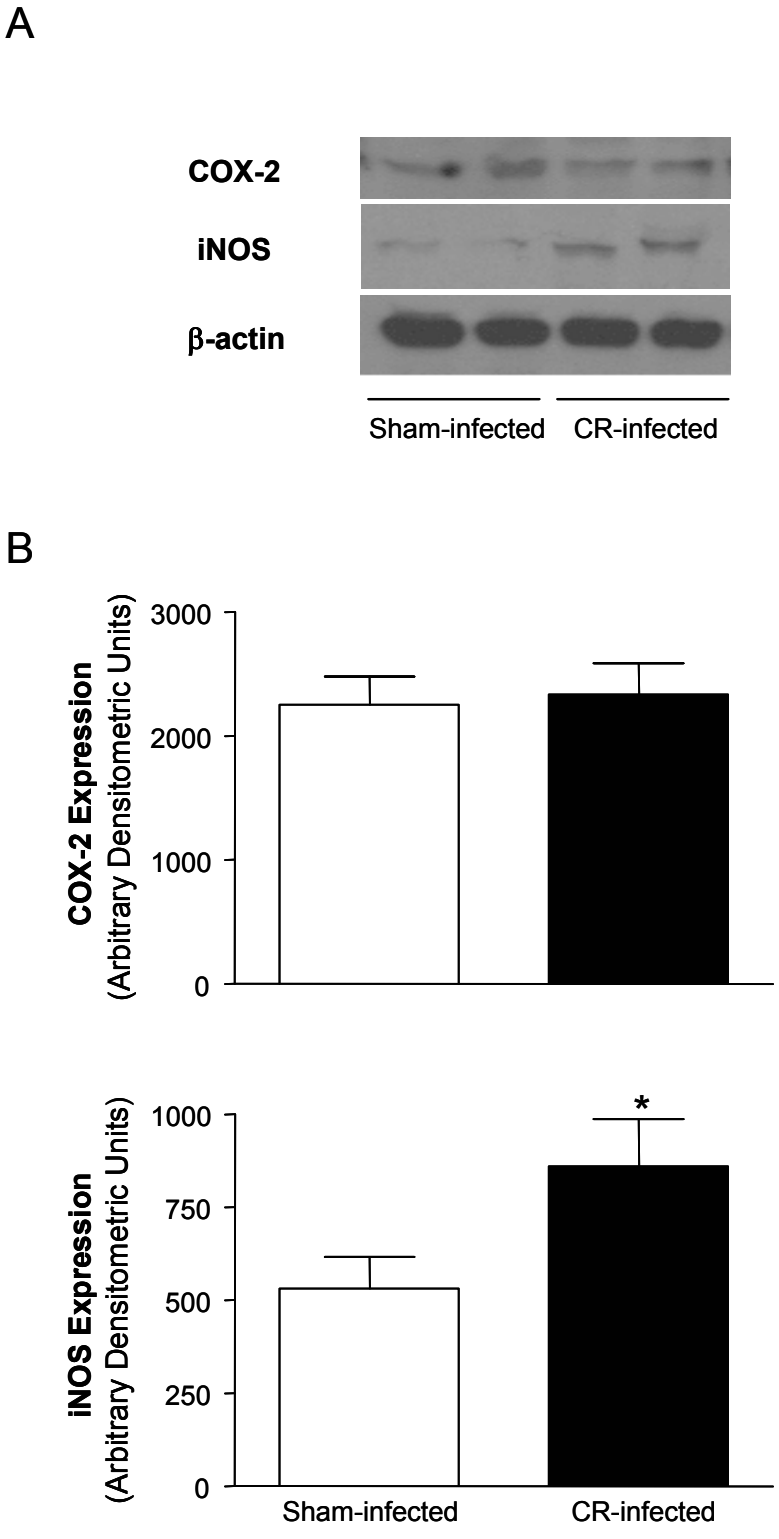

A
COX-2
iNOS
β-actin
Sham-infected
CR-infected
B
COX-2 Expression
(Arbitrary Densitometric Units)
3000
2000
1000
0
iNOS Expression
(Arbitrary Densitometric Units)
1000
750
500
250
0
*
Sham-infected
CR-infected

Figure 5.3 C.

Figure 5.3. Western blots and densitometry analysis for iNOS and COX-2 protein expression 10, 30 and 60 days following *Citrobacter rodentium* infection. A. 10 days following infection, significant increases in both iNOS and COX-2 protein expression are observed as compared to sham-infected animals. B. 30 days following infection, iNOS expression in infected animals remains elevated, whereas COX-2 expression has returned to control levels. C. 60 days following infection, iNOS expression remains elevated in infected animals versus sham-infected animals. * $p < 0.05$ vs. sham-infected mice. N=6-8.

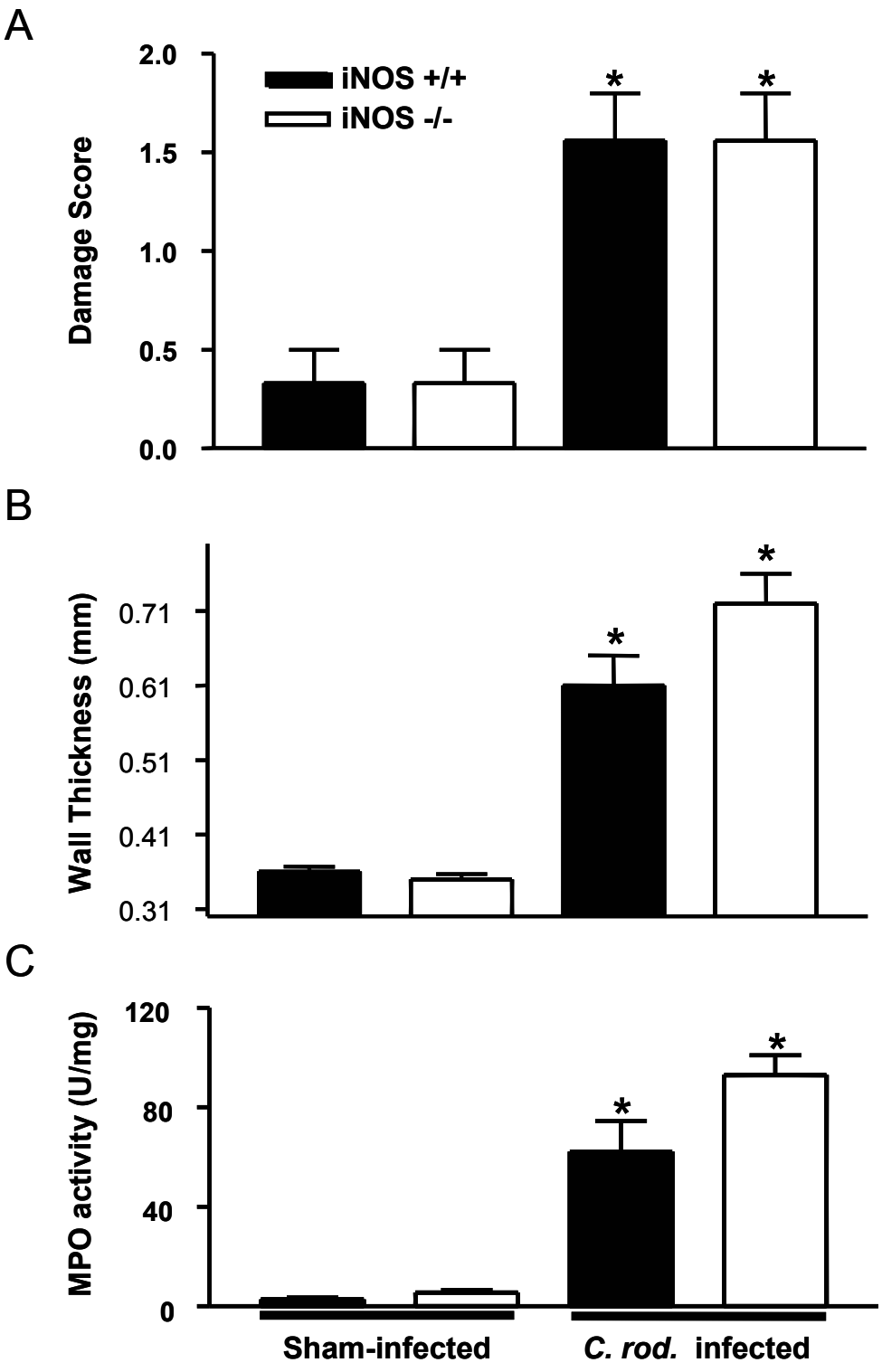

A
2.0
1.5
1.0
0.5
0.0
Damage Score
iNOS +/+
iNOS -/-
*
*
B
0.71
0.61
0.51
0.41
0.31
Wall Thickness (mm)
*
*
C
120
80
40
0
MPO activity (U/mg)
*
*
Sham-infected
C. rod. infected

Figure 5.4.

Figure 5.4. Inflammatory response in mouse colon following *Citrobacter rodentium* infection in iNOS-deficient and wild-type mice. The following inflammatory parameters were measured at 10 days following *C. rodentium* infection in iNOS-deficient and wild-type mice. A. Macroscopic damage scored according to the parameters in Chapter 2.4.1. B. Colon wall thickness measured using digital calipers. C. Myeloperoxidase activity in colonic tissue sections. * $p<0.05$ vs. sham-infected mice of same background. N=8.

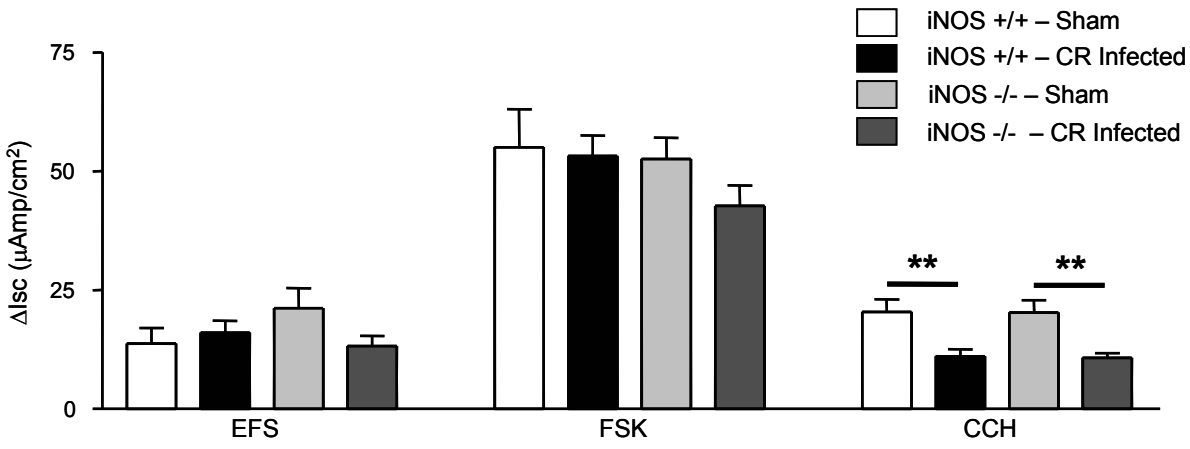


Figure 5.5. Changes in short circuit current by secretagogues following *Citrobacter rodentium* infection in iNOS-deficient and wild-type mice. At 10 days following *C. rodentium* infection, colons were removed and mounted in modified Ussing chambers and stimulated by electrical field stimulation (EFS; 50 V, 10 Hz, 5 s), forskolin (FSK; 10 μM) and carbachol (CCH; 5 μM). There was no significant difference in the change in short circuit current following EFS between infected and sham-infected animals. Infection did not evoke a significant difference in the FSK-induced change in short circuit current between infected and sham-infected animals. The addition of carbachol to tissues from infected mice caused a significant decrease in short circuit current compared to sham-infected mice of the same genotype. The same was observed in iNOS-deficient mice suggesting that iNOS-derived nitric oxide does not play a role in the altered response to carbachol following infection. ** $p<0.01$ vs. sham-infected mice. N=8.

C. rodentium infection resulted in increased damage score in both the rofecoxib and vehicle treated mice at 10 days compared to sham controls (Figure 5.6 A). However, there was no significant difference between the rofecoxib and vehicle treated *C. rodentium* infected animals. Treatment with rofecoxib reduced the increases in wall thickness (Figure 5.6 B) and MPO activity (Figure 5.6 C) caused by *C. rodentium* infection.

In Ussing chamber experiments, pretreatment with rofecoxib did not alter responses to electrical field stimulation, forskolin or carbachol (Figure 5.7). Rofecoxib was also administered acutely to colonic specimens mounted in Ussing chambers 10 days following sham or *C. rodentium* infection. Acute administration of rofecoxib did not affect the responses to secretagogues in any of the groups (Figure 5.8).

Two methods were employed to assess whether the rofecoxib treatment was causing differential infection between the groups. First, bacteria, cultured on MacConkey agar was recovered from rectal swabs from the infected mice, regardless of vehicle or rofecoxib treatment. Sham-infected animals had no recoverable bacteria. Second, fecal pellets were homogenized, plated on MacConkey agar and colony forming units were counted. The sham-infected animals, vehicle and rofecoxib treated, showed low numbers of colony forming units. (vehicle, 774.6 ± 193.4; rofecoxib, 386.1 ± 113.0 CFU/mg; $p > 0.05$) *C. rodentium*-infected mice, had increased numbers of CFU compared to the sham-infected mice but this was not different between vehicle and rofecoxib treatment groups (vehicle, 4336.8 ± 562.6; rofecoxib, 3735 ± 367.1 CFU/mg; $p > 0.05$).

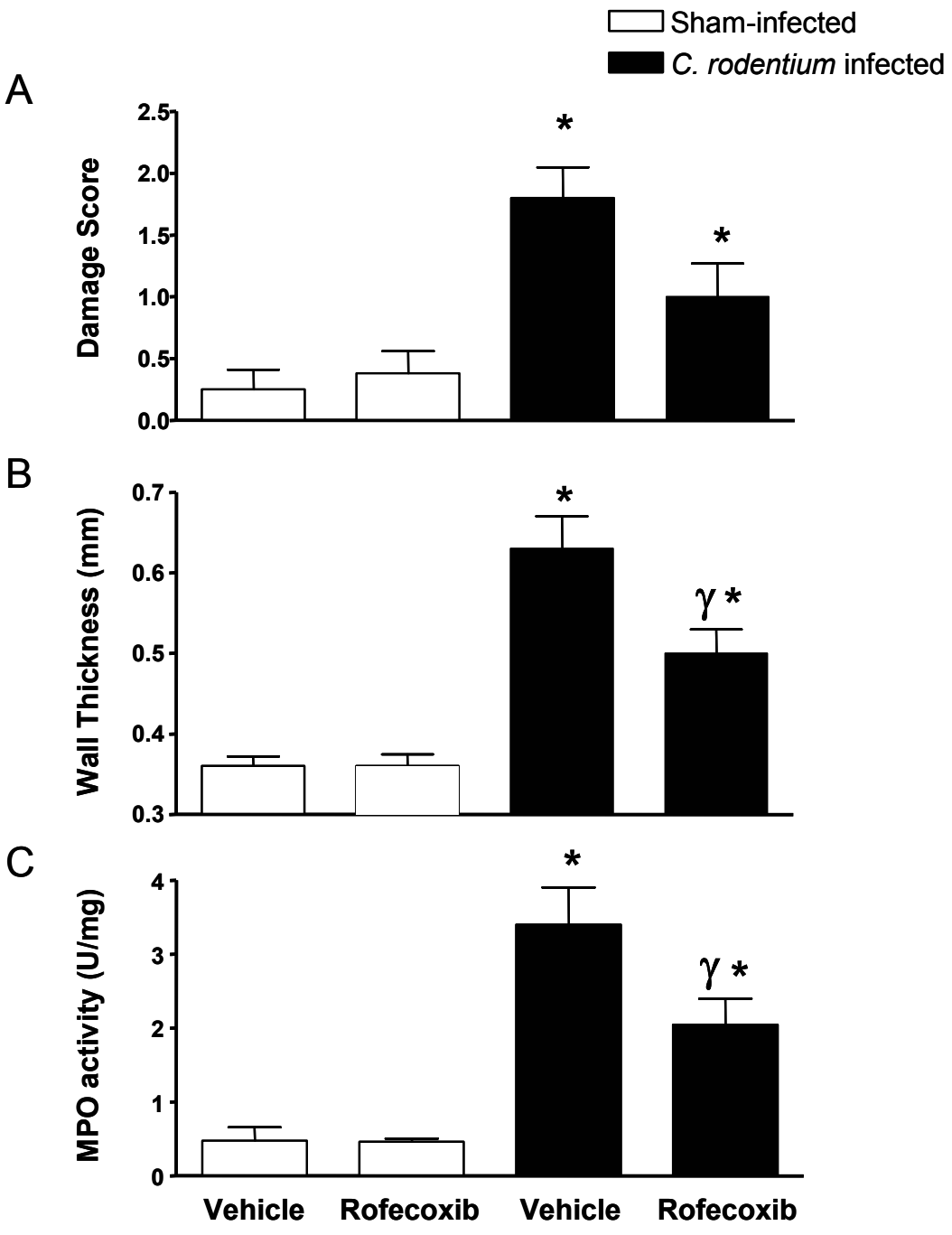

Sham-infected
C. rodentium infected
A
Damage Score
2.5
2.0
1.5
1.0
0.5
0.0
*
*
B
Wall Thickness (mm)
0.7
0.6
0.5
0.4
0.3
*
γ *
C
MPO activity (U/mg)
4
3
2
1
0
*
γ *
Vehicle
Rofecoxib
Vehicle
Rofecoxib

Figure 5.6.

Figure 5.6. Inflammatory response in mouse colon following *Citrobacter rodentium* infection in mice treated with rofecoxib or drug vehicle. The following inflammatory parameters were measured at 10 days following *C. rodentium* infection in mice treated with the COX-2 inhibitor rofecoxib (10 mg/kg/day) or drug vehicle. A. Macroscopic damage scored according to the parameters in Chapter 2.4.1. B. Colon wall thickness measured using digital calipers. C. Myeloperoxidase activity in colonic tissue sections. * $p < 0.05$ vs. sham-infected mice. $^{\gamma}$ $p < 0.05$ vs. vehicle treatment. N=8.

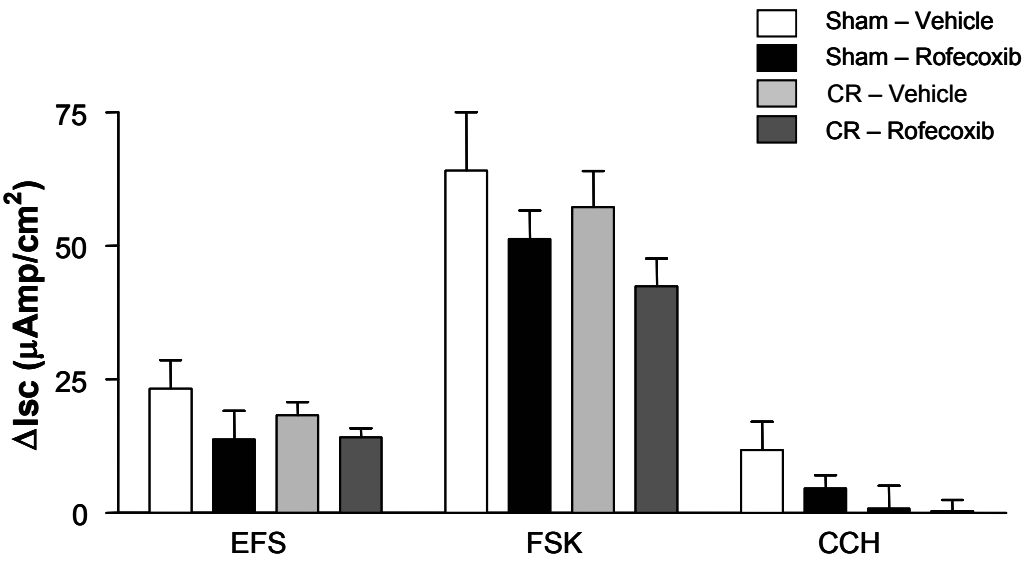


Figure 5.7 Changes in short circuit current by secretagogues following *Citrobacter rodentium* infection in mice treated with rofecoxib. At 10 days following *C. rodentium* infection and treatment with rofecoxib (10 mg/kg/day) or vehicle, colons were removed and mounted in modified Ussing chambers and stimulated by electrical field stimulation (EFS; 50 V, 10 Hz, 5 s), forskolin (FSK; 10 μM) and carbachol (CCH; 5 μM). There was no significant difference between groups in the change in short circuit current following any of the stimulations. N=6-8.

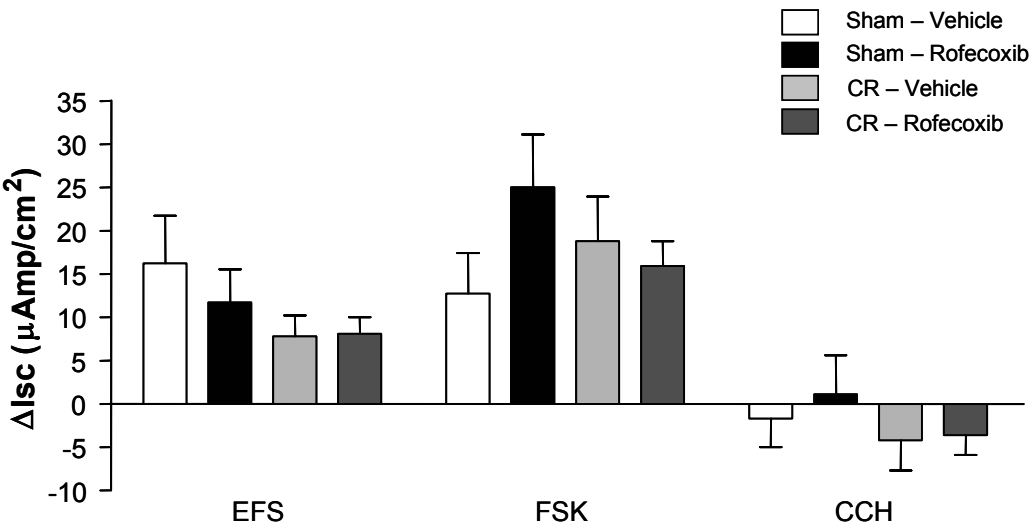


Figure 5.8. Changes in short circuit current by secretagogues following *Citrobacter rodentium* infection in mouse colons treated acutely with rofecoxib. At 10 days following *C. rodentium* infection, colons were removed and mounted in modified Ussing chambers and treated with rofecoxib (10 μM, 10 mins). Tissue was then stimulated by electrical field stimulation (EFS; 50 V, 10 Hz, 5 s), forskolin (FSK; 10 μM) and carbachol (CCH; 5 μM). There was no significant difference between groups in the change in short circuit current following any of the stimulations. N=6.

5.4 Discussion:

In this study, we have confirmed that *C. rodentium* infection in C57/Bl6 mice causes colitis and now show that the response to infection is associated with iNOS-derived NO-independent epithelial dysfunction. This infection was originally shown to cause A/E lesion formation and acute colitis (Barthold et al., 1978) and our findings support the latter as parameters of inflammation were significantly elevated at 7 and 10 days. Initial studies with *C. rodentium* infection in mice demonstrated an association with colonic crypt hyperplasia (Johnson and Barthold, 1979; Barthold, 1980). In this study, increased mucosal wall thickness was observed in infected mice as early as 7 days and remained significantly different even at 60 days post infection. Myeloperoxidase activity was elevated at 3, 7 and 10 days. This observation is consistent with previous studies that have shown increased neutrophil infiltration following *C. rodentium* infection (Higgins et al., 1999).

A/E lesion formation and the bacterial factors involved in *C. rodentium* infection have been well studied. The locus of enterocyte effacement (LEE) encodes a type III secretion system that directs the transport of bacterial proteins into the host cell (Hueck, 1998). The role of many of the effector proteins have recently been elucidated for both *E. coli* and *C. rodentium* due to strong homology between the genes. Other studies have focused on the mechanisms underlying the development of the inflammatory response to *C. rodentium* infection such as the role of B and T cells (Simmons et al., 2003). Despite recent studies aimed at expanding the knowledge of the effector proteins

involved and the inflammatory response to *C. rodentium* infection, little is known about functional changes. The results of this study are unique in that we use the *C. rodentium* model of pathogen/epithelium interactions and characterize it in terms of mucosal barrier and ion transport function.

A major component of epithelial barrier function is the secretion of ions such as chloride, creating osmotic pressure and thus the movement of water. This process is an important aspect of host defense since it prevents bacteria and other pathogens from colonizing on the epithelial surface as well as preventing bacteria, their products, or other antigens from crossing into the lamina propria. In previous studies, we have shown that in a rat model of TNBS colitis, inflammation induced a suppression of the response to cAMP-mediated secretagogues (MacNaughton et al., 1998). In contrast, in this model, the response to the adenylate cyclase activator, forskolin, did not elicit a significantly different secretory response between *C. rodentium* and sham-infected animals at any of the time points being studied. The same was observed for neurally-evoked secretion elicited by electrical field stimulation. However, the change in short circuit current following carbachol stimulation was reduced in infected animals. This carbachol-induced drop in short circuit current is consistent with what was observed in a DSS-induced model of colitis (Sayer et al., 2002). In that model, it was concluded that the decreased response to carbachol was dependent on a nicotinic receptor, revealed following DSS-induced colitis, which regulated cholinergic ion transport.

We have previously shown that in both the TNBS (MacNaughton et al., 1998) and radiation enteritis (Freeman and MacNaughton, 2000) mouse models, the hyporesponsiveness to cAMP-dependent secretagogues during inflammation was dependent on nitric oxide derived from iNOS. Six weeks following TNBS colitis in a rat model, this hyporesponsiveness was still observed even though colonic histology had returned to normal. Nevertheless, at this time point, iNOS was still elevated (Asfaha et al., 1999), and inhibition of iNOS during the recovery phase restored normal secretory function and prevented the increased bacterial translocation associated with hyporesponsiveness to secretagogues (Asfaha et al., 2001). iNOS has also been implicated as having effects on intestinal inflammation. Selective inhibition of iNOS with 1400W was shown to suppress the release of the inflammatory cytokines TNF-α and IL-6 in mucosal explants from patients with active UC (Kankuri et al., 2003). In the rat model of TNBS colitis, this same inhibitor reduces damage, edema, histological changes and MPO activity in animals treated with TNBS compared to control animals (Kankuri et al., 2001; Menchen et al., 2001). This prompted us to investigate if nitric oxide had a role in the bacterial translocation we observed in the model of *C. rodentium* infection.

In this study and consistent with previous studies, Western blotting revealed that iNOS expression was upregulated at 10, 30 and 60 days following *C. rodentium* infection. However, in contrast to the other colitis models, the increase in iNOS expression did not seem to be a key component in the development of inflammation following infection or in the ion transport properties.

When iNOS-deficient mice were infected with *C. rodentium*, there was no difference in damage score, wall thickness or MPO activity compared to wild-type animals. Similarly, colons from iNOS-deficient mice showed the same change in the reduction of the Isc response to carbachol following infection as in the wild-type animals. In the iNOS-deficient mice studies, while there was still a reduction in the change in short circuit current following carbachol stimulation in infected mice, there was not the complete current reversal as was observed in the initial 10 day studies. One possible explanation for this is that the mice for the two experiments were obtained from different sources, and therefore their native gut flora may have been different. The reason for choosing mice from a different source for the iNOS-deficient mice studies was to ensure that the knockout animals and wild-type controls were obtained from the same genetic background.

In the iNOS-deficient mice, there was also no difference in bacterial translocation to the mesenteric lymph nodes compared to wild-type controls following infection which also contrasts with other models of inflammation we have used previously. This suggests that the increase in iNOS expression and the presumed increase in nitric oxide production is not playing a role in disrupting epithelial barrier function. Taken together with the ion transport data, the colitis resulting from *C. rodentium* infection, the resulting decrease in barrier function, and increased bacterial translocation occur independently of iNOS activity. Previous studies have also shown that iNOS-deficient mice show no difference in tissue pathology following *C. rodentium* infection (Vallance et al., 2002). In these studies it was also shown that only 15% of cells infected with *C. rodentium*

express iNOS, whereas 85% of uninfected cells express iNOS. The authors suggest that perhaps *C. rodentium* has developed mechanisms to avoid exposure to nitric oxide derived by host cells (Vallance et al., 2002). Another possibility that must always be considered when using iNOS-deficient mice is that there are other nitric oxide synthases, eNOS and nNOS, which may compensate for the absence of nitric oxide derived from iNOS. Whether this is the case in our experiments remains to be determined.

Previous findings have demonstrated a role for COX-2 in prolonged epithelial secretory dysfunction (Zamuner et al., 2003). In these studies, six weeks after induction of TNBS colitis, colonic COX-2 and prostaglandin (PG) D_2 were elevated concomitantly with diminished colonic chloride secretory responses to EFS and the phosphodiesterase inhibitor IBMX. The use of a selective COX-2 inhibitor returned secretory function to normal and also prevented increased bacterial translocation. Exposing the colonic tissue to PGD_2 also caused diminished chloride secretory responses. In another study using the rat model of TNBS colitis, COX-2 inhibition had beneficial effects by diminishing the initial stages of inflammation (Martin et al., 2003). Based on these previous findings in a different model of colitis, we wanted to investigate the role of COX-2 in *C. rodentium* infection. Using Western blotting, we showed that COX-2 expression was increased 10 days following *C. rodentium* infection. However, in contrast to previous findings in TNBS colitis (Zamuner et al., 2003), COX-2 expression associated with *C. rodentium* infection returned to control levels at 30 and 60 days.

We then investigated if treatment with the selective COX-2 inhibitor, rofecoxib, would have any effects on barrier function and inflammation following infection. Treatment with rofecoxib or the vehicle control did not change the degree of infection as assessed by plating rectal swabs and fecal pellets at the end of treatment. Treatment with rofecoxib or vehicle did not commence until 3 days after infection to allow the infection to initiate and begin progressing normally. There were no changes in ion transport observed between the groups following electrical field stimulation, forskolin or carbachol stimulation. Overall macroscopic damage score was not different between the rofecoxib and vehicle treated infected mice, although they were both increased compared to sham-infected mice. Wall thickness was also observed to be increased compared to sham-infected controls for both rofecoxib and vehicle treated mice. However, the increase in wall thickness was not as prominent in the rofecoxib treated group. Similarly, when assaying for MPO activity, the rofecoxib treated infected group was less than the infected group treated with drug vehicle. These data suggest that COX-2 may have a partial role in the development of inflammation following *C. rodentium* infection, but is not solely responsible for the changes observed.

It is interesting that the mucosal changes and bacterial translocation observed following *C. rodentium* infection were not due to iNOS or COX-2 products since our previous findings in other models of intestinal inflammation suggested that these were likely candidates. Immune mediators such as cytokines have been studied previously, with IL-12, IFN-γ (Simmons et al., 2002) and TNF-α (Goncalves et al., 2001) increased following *C. rodentium* infection.

These studies demonstrated that mice deficient in these cytokines show increased pathology and increased susceptibility to systemic infection. While these studies have not addressed mucosal function such as changes in response to secretagogues, it is possible that they may alter barrier function during infection. For instance, IFN-γ has previously been shown to inhibit epithelial ion transport. In T84 cells, following exposure to IFN-γ, NKCC1 is downregulated resulting in a decreased response to chloride secretagogues (Colgan et al., 1994). T84 cells also demonstrated a decreased secretory response to forskolin and carbachol when cocultured with monocytes and activated T-cells, or cocultured with monocytes and activated T-cell conditioned media. A neutralizing antibody to IFN-γ prevented this response (McKay et al., 1996). Another possible area of study is with respect to the proteinase-activated receptors (PARs). PAR-2 in particular has been shown to be activated by serine proteinases present in the luminal fluid of *C. rodentium* infected mice (Hansen et al., 2005). PARs have also been implicated in changes in epithelial permeability (Chin et al., 2003) and may therefore play a role in the changes leading to increased bacterial translocation following infection. Further studies in these areas may lead to a better understanding of the mechanisms underlying the changes in mucosal function in infectious colitis.

In summary, our data demonstrate that *C. rodentium* infection causes changes to the colonic mucosa including increases in damage score, wall thickness, MPO activity, epithelial barrier function, iNOS and COX-2 expression and bacterial translocation. Many of these changes are consistent with other

experimental models of colitis indicating that not only can this model be used to study *in vivo* effects of A/E pathogens, but it can also serve as a reliable model for infectious colitis. However, the changes observed in this model appear to be independent of iNOS activity, and may be mediated, at least in part, by COX-2.

Chapter 6: *Citrobacter rodentium* infection and ionizing radiation:

6.1 Introduction:

The previous objectives dealt primarily with NO and barrier function in terms of both the unchallenged epithelium and the epithelium infected with a bacterial pathogen. At the outset, however, we described the current state of knowledge regarding the etiology of chronic inflammatory disorders and made the case for a luminal pathogen and bacterial translocation as a possible causative factor. Our next objective was to assess mucosal function, specifically barrier properties of the epithelium, in a model of known epithelial dysfunction combined with the challenge of an enteric pathogen. Based in part upon past experience and expertise with a murine model of radiation enteropathy, we assessed the effects of *C. rodentium* infection on the mucosal response to intestinal exposure to ionizing radiation.

Radiation-induced gastrointestinal symptoms are a major problem for patients undergoing radiotherapy for the treatment of cancer (Kao, 1995). In Canada, 38% of women and 44% of men will develop cancer in their lifetimes. Prostate cancer is the most prevalent cancer among men, and colorectal cancer is the second most prevalent cancer in both men and women (Statistics Canada 2005). Therefore, many patients require abdominopelvic radiotherapy. Even as modern day treatments improve, radiotherapy is a mainstay in the treatment of cancer and as a result, many patients will experience the radiation-induced

gastrointestinal symptoms that accompany this treatment when applied to the abdominopelvic region.

Intestinal epithelial dysfunction is a major complication following irradiation. As early as a few days post-irradiation, the ability of the intestinal epithelium to respond to secretagogues is diminished. This dysfunction is significant as the epithelium is the first line of host defense against luminal pathogens. Without this important aspect of the host defense system, pathogens are able to more readily cross into the lamina propria, and this can initiate or prolong an inflammatory response (Greger, 2000). In previous studies from our lab, it has been shown that exposure of mice to gamma irradiation leads to acute hyporesponsiveness to secretagogues (Freeman and MacNaughton, 2000) and increased expression of the inducible form of nitric oxide synthase (iNOS) (Freeman and MacNaughton, 2000; MacNaughton et al., 1998). Ionizing radiation has been known for some time to lead to bacterial translocation in both humans and animals (Rosoff, 1963; Guzman-Stein et al., 1989). It is also known that these acute complications can also lead to more serious chronic complications (Novak et al., 1979; Nussbaum et al., 1993; Saclarides, 1997). Therefore, it is of great interest to study other epithelial interactions that may lead to more serious complications following radiotherapy.

Previous studies in a rat model of abdominal irradiation have shown that a single dose of radiation causes bacterial translocation into the mesenteric lymph nodes (Guzman-Stein et al., 1989). In that study, there was 100% positive culture in mesenteric lymph nodes 12 hours to 4 days post-irradiation, however,

macroscopic signs of inflammation were not observed until 3 days post-irradiation with maximum damage on day 4. Bacterial translocation following irradiation may be responsible for the progression from acute to chronic enteritis. Since a luminal factor has been implicated in the development of inflammatory bowel disease (reviewed in Elson and Cong, 2002). Therefore it is reasonable to suggest that a luminal factor can also be involved in the severity of radiation enteritis.

Taken together, the observations that ionizing radiation leads to secretory dysfunction and bacterial translocation provide the rationale for the present study. We investigated a mouse model of irradiation combined with *C. rodentium* infection to examine the effects of both infection and irradiation on epithelial dysfunction and bacterial translocation. The findings in this study are clinically relevant to members of the population that require abdominopelvic radiotherapy. We demonstrate that the combined action of infection and irradiation produces an outcome with increased bacterial translocation and systemic infection greater than what would be expected if the results of each action on its own were additive.

6.2 Materials and Methods:

6.2.1 Animals and C. rodentium Infection:

Male C57Bl/6 mice (Charles River, Montreal, PQ) were used for these experiments and infected with *C. rodentium* according to the protocols described in Chapter 2.1.2. Mice were killed on day 10-13 post-infection.

6.2.2 Ionizing Radiation:

Three days prior to experiments, mice were transported in a RadDisk container (Braintree Scientific, Braintree, MA) and placed inside a Gammacell 40 Extractor irradiator (MDS Nordion, Kanata, ON). Mice were exposed to a cesium-137 source for the time required to deliver a whole body dose of 5 Gy as determined by the dose rate of the source. Control animals were placed in the irradiator for the same time period but were not exposed to the cesium-137 source.

6.2.3 Intestinal Inflammation:

Mice were killed by cervical dislocation. Following laparotomy, colons were removed and cut open longitudinally along the mesenteric border. Macroscopic damage and myeloperoxidase activity were assessed as described in Chapter 2.4.

6.2.4 Ion Transport Experiments:

Ion transport in mouse colon was measured in modified Ussing chambers as described in Chapter 2.3.

6.2.5 Assessment of Permeability:

Following establishment of baseline Isc, 6 μCi ^{51}Cr-EDTA was added to the serosal chamber. At time zero and at 25 mins, 100 μl samples were taken from both the serosal and mucosal sides. At 5, 10, 15 and 20 mins, samples were taken from the mucosal side. After each sample was taken, an equal

amount of Kreb's buffer was added to readjust the total volume in each chamber. Samples were read on a Cobra Series Auto-Gamma Counting System (Packard, Meriden, CT). Results were expressed as the percent ^{51}Cr-EDTA recovered on the mucosal side based on the initial amount added to the serosal side 25 minutes earlier.

6.2.6 Determination of Bacterial Translocation:

Bacterial translocation to the mesenteric lymph nodes, liver and spleen was determined as described in Chapter 2.5. Cardiac puncture was performed and blood was collected by syringe and plated onto MacConkey agar. The samples were incubated overnight at 37^{o}C, and bacterial CFU enumerated. The frequency of bacterial translocation into the blood was expressed as the proportion of mice showing positive colonization from blood samples.

6.3 Results:

6.3.1 Histological Damage and Inflammation

Upon laparotomy, in non-irradiated *C. rodentium* infected animals, edema of the colon was observed, whereas erythema was not. In the irradiated *C. rodentium* infected animals, edema of the colon was observed with some animals also showing erythema and diarrhea. Some of the irradiated sham-infected mice also showed erythema but no edema. The non-irradiated sham-infected animals had no macroscopic damage. Statistically, the macroscopic damage score was significantly higher in both the non-irradiated *C. rodentium* infected animals, as well as the animals that were both infected and irradiated compared to sham-

infected non-irradiated animals. There was also a significant difference in damage score (Figure 6.1 A) and colonic wall thickness (Figure 6.1 B) between the sham-infected and *C. rodentium*-infected groups that were exposed to 5 Gy irradiation. Myeloperoxidase activity was significantly elevated in non-irradiated *C. rodentium*-infected animals compared to non-irradiated sham-infected animals (Figure 6.1 C). There was no significant difference in myeloperoxidase activity between infected and non-infected animals in groups exposed to 5 Gy irradiation.

6.3.2 Ion Transport Experiments:

Ion transport experiments were performed to assess if the morphological changes associated with *C. rodentium* infection and/or irradiation resulted in changes in net electrogenic ion flux. Conductance of the tissue was not significantly different between any of the groups. Electrical field stimulation, forskolin, and carbachol caused immediate and transient increases in short circuit current. There was no statistical difference in the change in short circuit current between infected and sham-infected animals receiving 0 Gy irradiation. Likewise, infected or sham-infected animals receiving 5 Gy irradiation showed no statistical difference in change in short circuit current (Figure 6.2).

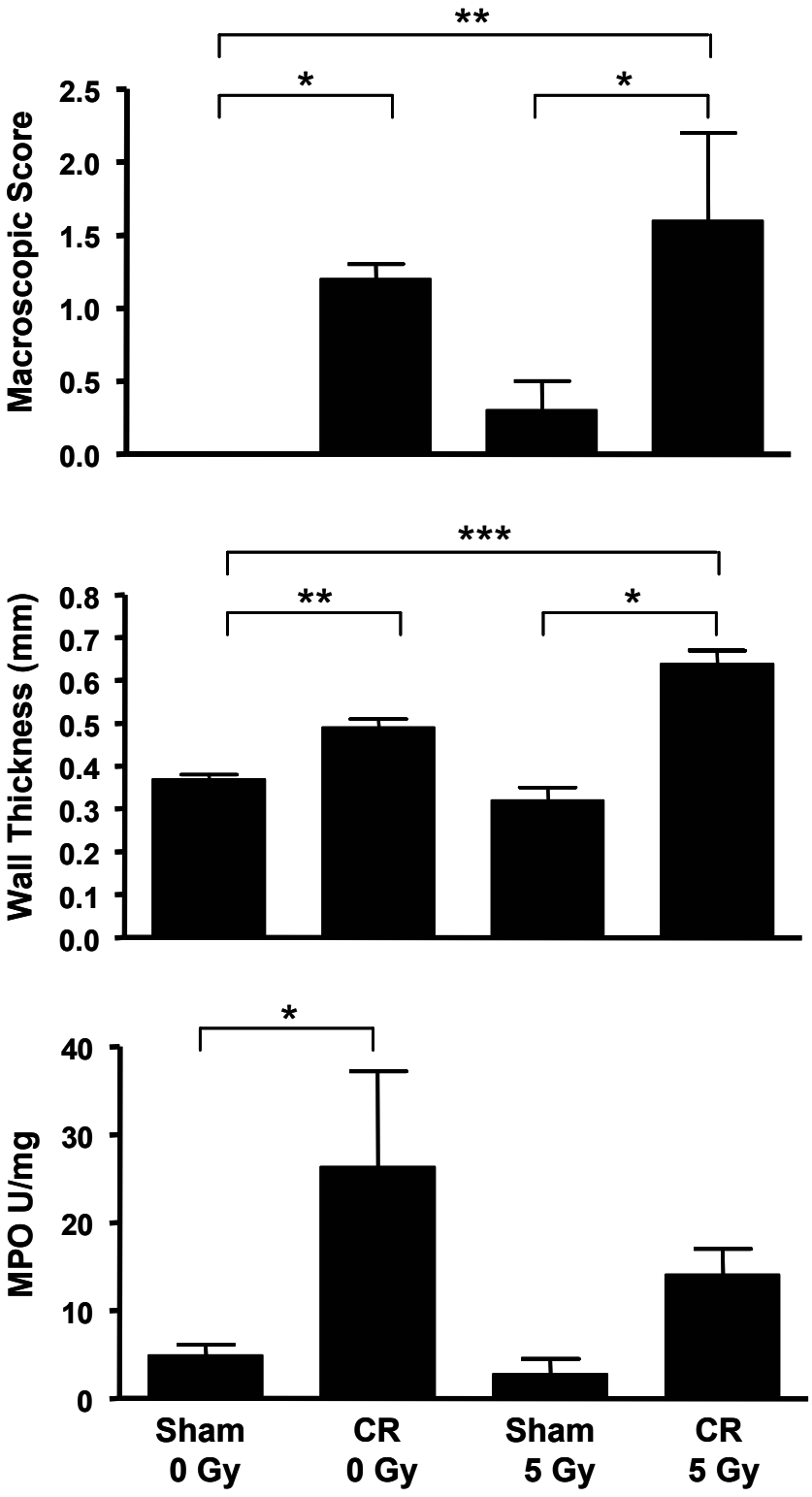

**
*
*
Macroscopic Score
2.5
2.0
1.5
1.0
0.5
0.0
***
**
*
Wall Thickness (mm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
MPO U/mg
40
30
20
10
0
Sham
0 Gy
CR
0 Gy
Sham
5 Gy
CR
5 Gy

Figure 6.1.

Figure 6.1. Inflammatory response in mouse colon following *Citrobacter rodentium* infection in irradiated and non-irradiated mice. The following inflammatory parameters were measured at 10 days following *C. rodentium* (CR) or sham infection in irradiated (5 Gy) and non-irradiated (0 Gy) mice. A. Macroscopic damage scored according to the parameters in Chapter 2.4.1. B. Colon wall thickness measured using digital calipers. C. Myeloperoxidase activity in colonic tissue sections. * $p<0.05$, ** $p<0.01$, ***$p<0.001$ between groups. N=16.

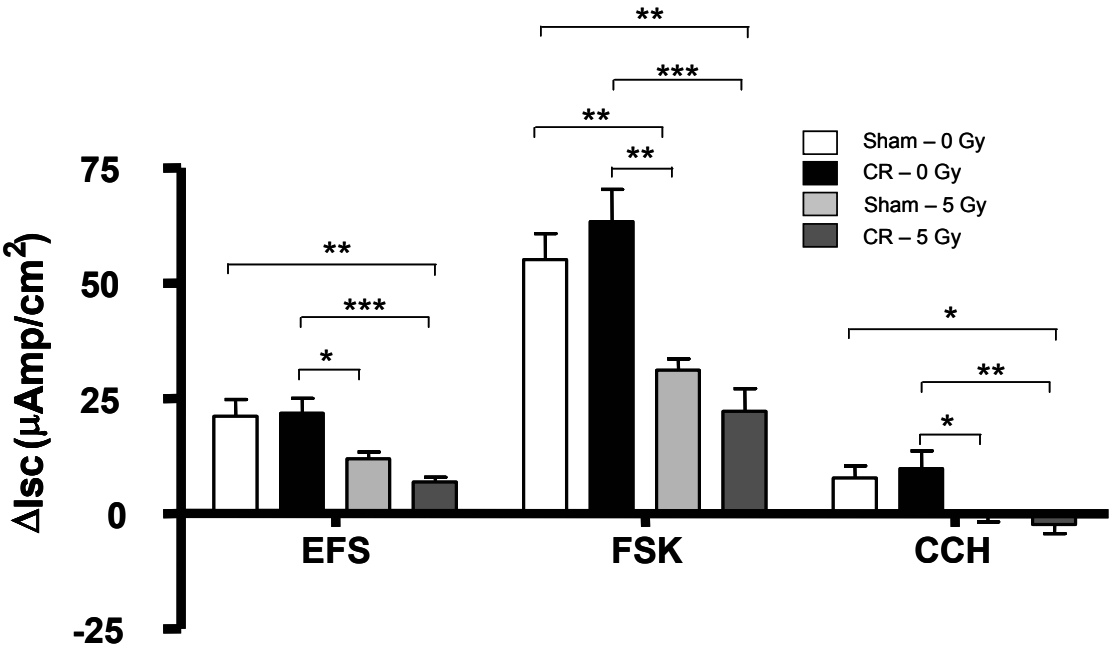


Figure 6.2. Changes in short circuit current by secretagogues following *Citrobacter rodentium* infection in irradiated and non-irradiated mice. At 10-13 days following *C. rodentium* (CR) infection, and 3 days following irradiation (5 Gy), colons were removed, mounted in modified Ussing chambers and stimulated by electrical field stimulation (EFS; 50 V, 10 Hz, 5 s), forskolin (FSK; 10 μM) and carbachol (CCH; 5 μM). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ between groups. N = 15.

6.3.3 Bacterial Translocation:

Homogenates of liver, spleen and mesenteric lymph nodes were negative for bacteria at the time points tested in sham-infected animals receiving 0 Gy or 5 Gy irradiation (Figure 6.3). Infected mice receiving 5 Gy irradiation showed significantly higher incidence of bacterial translocation than infected mice receiving 0 Gy irradiation. This group also showed a higher incidence of bacteria cultured from blood samples (Table 6.1).

6.3.4 ^{51}Cr-EDTA Permeability:

Prior to the various stimulations performed in the Ussing chambers, ^{51}Cr-EDTA was added to the serosal chamber bath. Samples were taken at five minute intervals and after 25 minutes, the percentage of ^{51}Cr-EDTA crossing the colonic tissue was assessed. There was no statistical difference in the amount of ^{51}Cr-EDTA recovered in the mucosal chamber bath between groups at this time point suggesting that there were no changes in colonic permeability (Table 6.2).

6.4 Discussion:

In this study, we have shown that in C57/Bl6 mice, *C. rodentium* infection, followed by ionizing radiation leads to significant increases in bacterial translocation and systemic infection as compared *C. rodentium* infection or irradiation alone. These changes occur without any significant differences to secretory function between irradiated sham-infected and irradiated *C. rodentium*-infected animals. Also, these results are independent of any changes in epithelial permeability as measured by the ^{51}Cr-EDTA technique.

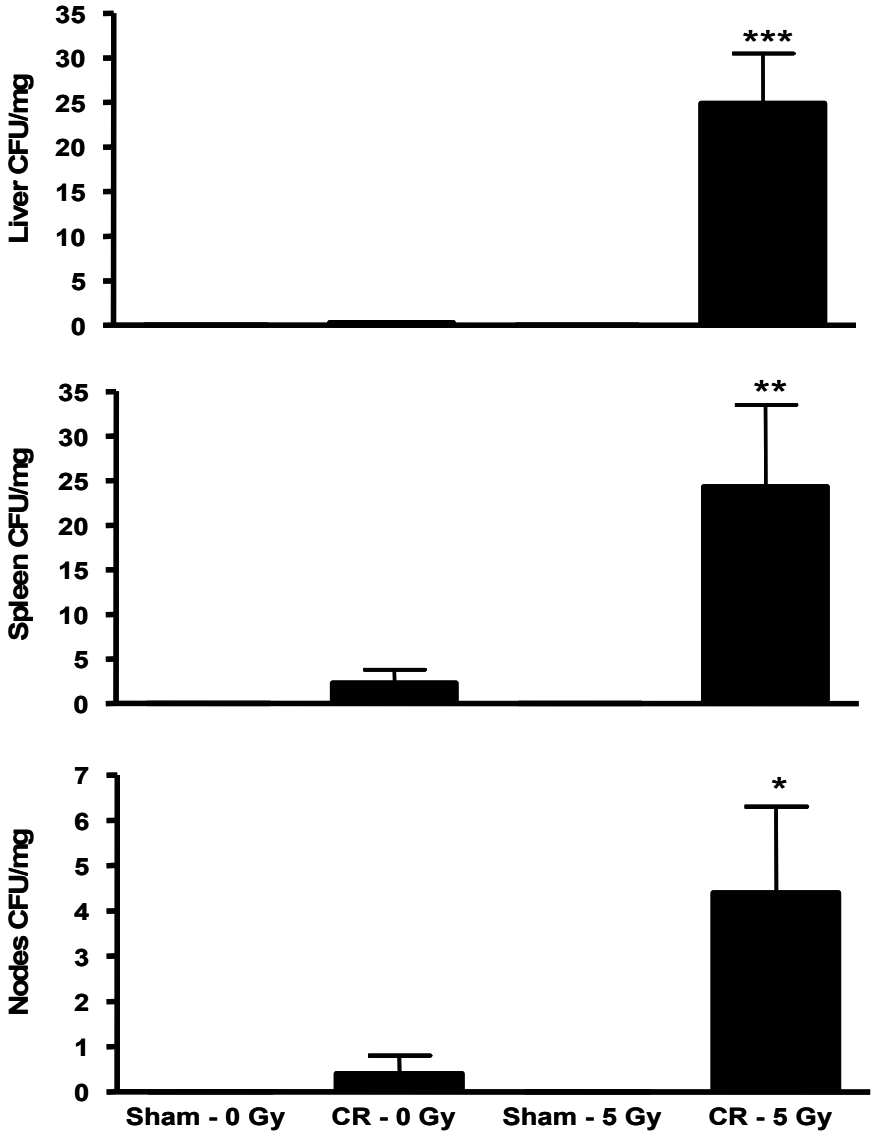


Figure 6.3. Bacterial translocation in the mesenteric lymph nodes, spleen and liver 10 days following *Citrobacter rodentium* infection and/or irradiation. Bacterial translocation was identified as the presence of colonies following plating of A. liver, B. spleen, and C. mesenteric lymph node homogenates on MacConkey agar and expressed as CFU/mg tissue. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. sham-infected irradiated animals. N=16.

Group	Incidence of bacteria recovered in the blood (number of animals)
Sham-infected – 0 Gy	0 of 16
CR-infected – 0 Gy	1 of 16
Sham-infected – 5 Gy	0 of 16
CR-infected – 5 Gy	10 of 16

Table 6.1. Incidence of bacteria cultured from blood 10 days following *Citrobacter rodentium* infection and/or irradiation. Bacterial translocation was identified as the presence of colonies following plating of the blood on MacConkey agar. The ratios listed indicate the number of animals showing translocation over the total number of animals in each group. N=16.

Group	% of initial ^{51}Cr-EDTA recovered in mucosal compartment after 25 mins
Sham-infected – 0 Gy	0.001584 ± 0.000172
CR-infected – 0 Gy	0.001410 ± 0.000131
Sham-infected – 5 Gy	0.001347 ± 0.000215
CR-infected – 5 Gy	0.001691 ± 0.000410

Table 6.2. ^{51}Cr-EDTA flux as a measure of colonic permeability 10 days following *Citrobacter rodentium* infection and/or irradiation. Values expressed for each group represent the percent of ^{51}Cr-EDTA recovered in the mucosal chamber as compared to the original amount added to the serosal chamber after 25 minutes. N=8.

While our findings in the previous chapter described inflammatory parameters and ion transport function associated with *C. rodentium* infection, the experiments reported in this chapter focused on the combination of infection and exposure to ionizing radiation. Consistent with our studies in the previous chapter, infection with *C. rodentium* increased macroscopic damage score, wall thickness and myeloperoxidase activity compared to sham-infected controls. Macroscopic damage score and wall thickness were also significantly increased in infected animals exposed to 5 Gy irradiation compared to both sham-infected non-irradiated and sham-infected irradiated animals. Myeloperoxidase activity was not significantly elevated in infected animals exposed to irradiation, consistent with our previous findings that whole body irradiation, such as that used here, results in significantly lower white blood cell counts and no inflammatory response (Freeman and MacNaughton, 2000).

Hyporesponsiveness to secretagogues occurs following exposure to ionizing radiation (Freeman and MacNaughton, 2000). The findings in this study are consistent in that animals exposed to 5 Gy irradiation showed significantly reduced responses to electrical field stimulation and forskolin. Interestingly, the response to carbachol in infected non-irradiated animals in this study did not show the reversal in short circuit current that was observed in the previous chapter. However, we still observed similar mucosal damage in the *C. rodentium* infected animals in both studies suggesting that the differences observed in the change in carbachol response do not appear due to differences in the degree of damage. These discrepancies will be discussed in more detail in Chapter 7.

In the present study, and consistent with our previous work with *C. rodentium*, we were able to culture bacteria on MacConkey agar from the mesenteric lymph nodes of infected animals although the CFU/mg were not significantly higher from sham-infected controls. When we infected and exposed the mice to 5 Gy irradiation, however, there were significant increases in bacteria numbers in the mesenteric lymph nodes, spleen, and liver. Bacteria were also cultured from the blood of infected irradiated animals indicating systemic infection. In sham-infected irradiated animals, however, bacteria were not cultured from the organ or blood samples. Since growth on MacConkey agar indicates a gram-negative lactose fermenter which may include a number of different species, it cannot be concluded from these studies that it is *C. rodentium* translocating across the epithelium. However, these findings still point to a situation whereby the combined action of both *C. rodentium* infection and ionizing radiation cause a dramatic increase in bacterial translocation leading to systemic infection. Interestingly, there is no difference in epithelial permeability between any of the groups being studied indicating that the bacterial translocation is occurring by a mechanism other than generalized colonic tissue permeability changes. In a previous study investigating the effects of abdominal radiation in rats, bacterial translocation to the mesenteric lymph nodes was also observed in this model at early time points without macroscopic histological changes (Guzman-Stein et al., 1989). While that study did not look specifically at epithelial permeability, the observation that mucosal integrity is still intact at early

time points post-irradiation would suggest that bacteria are able to move across the epithelium independently of barrier damage.

Evidence for a luminal factor being implicated for intestinal complications following irradiation has been suspected for some time, yet not extensively studied. In a guinea pig model of acute radiation enteritis, surgical bypass of the small bowel resulted in a segment isolated from luminal contents (Mulholland et al., 1984). In this empty intestinal segment, there was less damage and more crypt cells than in the irradiated segment containing luminal contents. In another study, it was shown that intestinal bacteria lead to increased lethality in rats following exposure to radiation (Rosoff, 1963). In those experiments, rats were treated with the non-absorbable antibiotic neomycin sulfate to eliminate *E. coli* and coliform bacteria from the lumen. The animals treated with neomycin showed improvement in body weight and 30-day survival compared with non-treated animals. Mice that were given neomycin for 20 days showed a 100% survival rate, compared to 20% for those treated for 3 days. Systemic administration of antibiotics was not protective indicating that it is a luminal bacteria involved in the development of radiation enteropathy (Rosoff, 1963). Our findings in this study support these earlier findings in that *C. rodentium* is a luminal bacterium and infection with this pathogen prior to irradiation causes significant increases in bacterial translocation leading to systemic infection.

Irradiation is also known to affect the immune response, and this can have an impact on bacterial infection. In a recent study (van Diepen et al., 2005), mice were infected with *S. enterica* and allowed to completely recover from infection

before being irradiated. Irradiated mice showed decreased $CD4^+$ T cells along with a secondary infection peak demonstrated by *S. enterica* being recovered from the spleens and livers of irradiated animals. T cell depletion induced by injecting anti-CD4 antibody also showed similar results in generating a secondary infection peak. This study shows that $CD4^+$ T cells are involved in suppression of *S. enterica* during latency. Both B cells and T cells have been shown to be critical in the clearance and development of immunity to *C. rodentium*. This role was first observed in mice depleted of B cells or $CD4^+$ T cells that were unable to resolve infection by *C. rodentium* and developed severe colitis (Simmons et al., 2003). Another study demonstrated the role for B cells in clearing *C. rodentium* infection and further showed that although B cells are required, absence of secretory IgA or IgM are not involved in host defense against this pathogen (Maaser et al., 2004). Since radiation is known to deplete lymphocytes (Berry and Denekamp, 1989), it is possible that the synergistic increase in bacterial translocation that we observed in this study following infection and irradiation treatment is dependent on immune cells but further experiments to confirm this are needed.

The above results present interesting and unique findings. The differences in inflammatory parameters being studied are dependent on *C. rodentium* infection but not irradiation. The differences in short circuit current in response to secretagogues is dependent on irradiation but not *C. rodentium* infection. However, the action of these treatments together showed significant differences in bacterial translocation and systemic infection. In a clinical setting

where patients are undergoing abdominopelvic radiotherapy, these findings suggest that the complication of bacterial infection may lead to more severe gastrointestinal symptoms and increase the chances of developing serious, chronic complications.

Chapter 7: General Discussion and Future Directions:

In addition to its role in nutrient and water absorption, the intestinal epithelium is also a first line of defense against bacteria and other potentially harmful materials present in the gut lumen. The epithelium acts as a barrier to prevent these pathogens from translocating into the intestinal mucosa. Tight junctions, which maintain a low level of epithelial permeability and secretion of ions to drive fluid movement, are two aspects of the epithelium which maintain barrier function.

Inflammatory diseases of the intestine are relatively common, yet their exact cause and mechanism are still unknown. Along with a genetic component that has been implicated in Crohn's disease, a luminal factor is also likely to play a role in the inflammation associated with this disease. Similarly, intestinal inflammation following exposure to ionizing radiation in the treatment of cancer has also been shown to involve secretory dysfunction and bacterial translocation that also involves a luminal pathogen component. In these and other inflammatory conditions, NO has been shown to be involved in regulating the secretory dysfunction leading to symptoms of clinical disease.

It was hypothesized that NO inhibits intestinal epithelial chloride secretion, thus allowing bacterial translocation that contributes to enteritis following irradiation. The major findings support this hypothesis in part and are outlined below.

1. NO inhibited cAMP-dependent CFTR trafficking in intestinal epithelial cells. This inhibitory effect was not mediated by pretreatment with the guanylate cyclase inhibitor, ODQ. This effect was transient and correlated temporally with changes in short circuit current in Ussing chamber studies.
2. NO disrupted the tight junction protein ZO-1, but not occludin, claudin-4 or claudin-5 in intestinal epithelial cells. This disruption did not lead to changes in epithelial permeability or bacterial translocation across epithelial monolayers.
3. *C. rodentium* caused colitis and iNOS-derived NO-independent epithelial dysfunction in C57/Bl6 mice. Infection caused significant reductions to the epithelial secretory response to carbachol at 3 and 7 days post-infection. Treatment with a COX-2 inhibitor resulted in reduced wall thickness and MPO activity at day 10 compared to vehicle-treated infected animals, but these changes were not associated with changes in ion transport.
4. *C. rodentium* infection combined with exposure to ionizing radiation resulted in significantly greater bacterial translocation and systemic infection than would be expected if each action on its own were additive. Bacterial translocation occurred without detectable changes to colonic permeability.

Together, these findings add substantially to our understanding of intestinal inflammation at both the cellular and whole animal levels. Previous studies have shown that NO is implicated in the hyporesponsiveness to secretagogues during inflammation. While it was shown that NO could directly

regulate isoforms of adenylate cyclase, thus reducing intracellular cAMP concentrations, it was not known if NO could also have a direct effect on the trafficking of CFTR to the apical membrane. In this study, we have shown that CFTR trafficking is inhibited by a NO donor in an epithelial cell line and this is also correlated to a reduction in the chloride secretory response following stimulation with forskolin. The concentration of the NO donor used in these studies was also shown not to cause changes in epithelial permeability or bacterial translocation.

In future studies, it would be interesting to determine if other NO donors acting over longer time periods would have different effects on tight junction proteins leading to changes in permeability and bacterial translocation. While the experiments in this study confirmed that the changes we observed in our trafficking and ion transport experiments were not due to changes in permeability, it would be interesting to see if varying the delivery of NO does lead to changes in epithelial permeability. Further, since we showed that only ZO-1 was disrupted following exposure to a NO donor, studies aimed at identifying the pathway for this specificity would provide a better understanding of tight junctional changes leading to increased epithelial permeability.

In addition to secretory dysfunction, a compromise in permeability can potentially lead to pathogens such as bacteria translocating into the lamina propria. Since a luminal factor has been shown to have a role in diseases of the intestine, such as Crohn's disease and radiation enteritis, we wanted to investigate functional changes following infection by an intestinal pathogen.

Although an increase in iNOS protein expression was observed following infection with *C. rodentium*, changes in ion transport were not dependent on this increase.

In other models of intestinal inflammation, increased iNOS expression has been associated with secretory hyporesponsiveness. Previous studies in the TNBS (Asfaha et al., 1999) and DSS (Green et al., 2004) models of colitis, and a mouse model of acute radiation enteritis (Freeman and MacNaughton, 2000) have demonstrated the NO-dependent nature of this dysfunction. However, in the present study using *C. rodentium* as an infectious model of colitis, we do not see the same secretory dysfunction despite increases in iNOS protein expression. Other studies using *C. rodentium* infection have observed increases in iNOS mRNA expression in the colons of infected animals (Vallance et al., 2002). In those studies, iNOS-deficient mice showed a slight delay in the clearance of infection, however, tissue pathology was not affected. Looking specifically at iNOS expression in epithelial cells following *C. rodentium* infection, 85% of uninfected cells expressed iNOS, whereas less than 15% of infected cells expressed iNOS. From these data, along with our current findings in which changes in ion transport following infection are independent of iNOS expression, we can speculate on the mechanisms accounting for these differences between models. First, since *C. rodentium* and other A/E pathogens are able to remain bound to epithelial cells and are present in the lumen for some time following infection, they may have evolved mechanisms to avoid NO-mediated antimicrobial defenses. Perhaps a specific effector protein secreted by *C.*

rodentium negates the effects of increased iNOS expression either by scavenging the NO produced or by regulating NO production by the enzyme itself. Second, the cytoskeletal rearrangements caused by A/E pathogen infection may prevent the recruitment of iNOS to the apical membrane since this event is associated with the actin cytoskeleton (Vallance et al., 2002). Further studies in these two areas may provide additional insight into the differences in secretory dysfunction between various experimental models of colitis that all show increased iNOS expression.

Additional future studies should investigate the mechanisms behind the functional changes seen in the mouse model of *C. rodentium* infection. For instance, in the studies reported here, there were some differences in the secretory response to carbachol following *C. rodentium* infection between different groups of animals. While these studies have not addressed the discrepancy between the positive versus negative change in short circuit current following carbachol stimulation, some explanations for these differences can be provided. As stated in Chapter 5, the mice for the experiments involving iNOS-deficient mice were obtained from a different source (Jackson Labs) than the other experiments (Charles River) since we wanted to use the same background strain and control for differences in mice between suppliers. It is possible that the microflora of the mice may differ between suppliers which may cause the mice to have varying responses to secretagogues. Indeed, the mice from Jackson labs showed larger changes in short circuit current for all stimulations performed in the Ussing chambers compared to the mice obtained from Charles

River. Perhaps this overall increase “masked” the effect that we were observing in the current reversal in response to carbachol. This is possible if the positive and negative responses are driven by different mechanisms as observed in other models of colitis (Green et al., 2004).

While the above explanation may account for the differences observed between the initial studies in Chapter 5 and the iNOS-deficient mice experiments, a discrepancy still exists between the results of carbachol stimulation in Chapter 5 and Chapter 6 in mice from the same supplier. Stress may have been a factor in these differences for two reasons. First, the mice in Chapter 6 were moved from the biohazard facility to the irradiator, even if they were not being exposed to irradiation. Second, the University of Calgary Health Sciences Centre was undergoing large amounts of construction during the time of these experiments and the excess noise and vibrations may have also led to additional stress for the animals. One final note to consider for all experiments is that since they were done on different dates, the biohazard facility itself may have contained additional pathogens contributing to changes in commensal microflora and thus altering the outcomes of experiments. However, groups of animals being compared in all studies were used for experimentation in the same time frame which still justifies comparing the results between experimental groups.

These possibilities for the discrepancies aside, further investigation into the mechanisms resulting in a positive versus negative change in short circuit current following carbachol stimulation may lead to better characterization of the processes involved in this infection and their role in host defense. Other recent

studies have investigated the role of proteinase-activated receptors (PARs) and *C. rodentium* infection. These studies implicate PAR-2 in the development of infectious colitis (Hansen et al., 2005) and may also block the reversal of the carbachol response that we observed in some of our studies.

We also investigated the effects of ionizing radiation combined with *C. rodentium* infection with respect to ion transport, epithelial permeability and bacterial translocation. Interestingly, the combined effect of infection and irradiation led to significantly higher increases in bacterial translocation and systemic infection than from infection or irradiation alone. However, these increases were not accompanied by changes in colonic permeability suggesting that bacteria are able to move into the lamina propria even in the absence of generalized decreases in mucosal barrier integrity.

It would also be interesting to more accurately determine the bacterial species involved in bacterial translocation and systemic infection following *C. rodentium* infection and exposure to ionizing radiation. Experiments in germ-free conditions or transfecting *C. rodentium* with an antibiotic resistance gene would confirm that it is *C. rodentium* translocating. Alternatively, PCR amplification of a *C. rodentium* specific gene could also be performed on the recovered colonies to differentiate the presence of *C. rodentium* versus other bacteria. Perhaps just as interesting, however, would be if the combined effects of *C. rodentium* infection and exposure to irradiation enhanced bacterial translocation for numerous commensal bacterial species.

Since some contend that conditions such as Crohn's disease represent a loss of immunological tolerance to normally innocuous luminal flora (Elson et al., 2002), another direction for future experiments would be the combined effects of *C. rodentium* infection and irradiation in iNOS-deficient animals or with the use of iNOS inhibitors. Although the findings in this study do not implicate iNOS in the changes in ion transport following *C. rodentium* infection, based on our previous findings, we speculate that the loss of iNOS expression or function would decrease bacterial translocation following the combined treatment of infection and irradiation. Since we have previously shown that exposure to ionizing radiation reduced total white cells, and clearance of *C. rodentium* infection is dependent on B-cells and $CD4^+$ T-cells, further investigation of these immune cells may also provide information about the mechanisms involved during combined infection and irradiation. Such an approach could reveal new avenues for therapeutic management in the clinical setting.

Another aspect of the effects of ionizing radiation that should be studied in the future is the long-term effects of exposure to ionizing radiation. While it is important to study the acute dysfunction as we have done in this study, ultimately we would like to investigate chronic enteritis and the mechanisms involved. Along with these studies would be the investigation of the processes involved with the shift from acute enteritis following radiation to development of chronic disease. This last point is of great clinical interest since although many patients receiving radiation therapy overcome the acute symptoms of enteritis, a subset of the population will develop chronic disease. While a luminal factor has been

implicated in radiation enteritis, specific mechanisms underlying this disease remain to be studied.

The future directions for experiments suggested above will help us better understand the role of secretion and barrier function in host defense against a luminal pathogen. While it has previously been shown in other models of colitis that secretory function can be restored through the loss of iNOS activity, this is not the case for *C. rodentium* infection. We have shown in this study that bacterial translocation following *C. rodentium* infection can occur, but that this process is significantly increased following exposure to ionizing radiation. Therefore, the next step is to determine whether the loss of iNOS activity can prevent bacterial translocation following infection and exposure to ionizing radiation. It will also be important from a clinical perspective to investigate the effects of bacterial infection when epithelial secretory dysfunction due to colitis or radiation enteritis is ongoing. We speculate that since the epithelium is in a state of impaired barrier function, infection may result in more severe complications. These studies may also help in determining the factors involved in progression from acute enteritis following radiotherapy to the development of chronic disease.

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