ABSTRACT

NIGHOT, MEGHALI PRASHANT. Role of ClC-2 and MAPK in Gastric Barrier Function. (Under the direction of Anthony Blikslager).

The gastric barrier is composed of a single layer of columnar epithelium, mucus and bicarbonate layer, and the intraepithelial tight junctions residing at the apical-most region of the paracellular space. The gastric barrier serves as the first line of defense against a hostile luminal environment. Once the epithelial barrier is disrupted, epithelial repair mechanisms must rapidly re-form a continuous epithelial monolayer and seal paracellular tight junctions in order to prevent entry of protons and bacteria. We studied the role of chloride channel ClC-2 and MAPK proteins in the gastric barrier function. We designed, developed, and optimized in vivo acid-injury and in vitro NSAID-injury model for our studies. We found that ClC-2 activation protects gastric mucosal barrier dysfunction by preventing disruption of tight junction protein occludin in both in vivo acid injury in pig gastric mucosa as well as in vitro NSAID injury in gastric epithelial cells. In parallel studies based on genetically modified mice, we found altered acid secretion, reduced H⁺K⁺ATPase expression, reduced parietal cell population, and disorganization of gastric glands in the ClC-2 knockout mice. Thus ClC-2 appears to have a critical role in the proper differentiation and organization of gastric glands.

In another piece of study we investigated the role of MAPK (Mitogen Activated protein Kinases) in gastric epithelial barrier function. We detected p38, and JNK, but not ERK MAPK phosphorylation following indomethacin-induced injury in the gastric cell line.
In further analyses of TJ proteins, expression of occludin was found to be reduced by indomethacin and inhibition of p38 MAPK prevented the indomethacin-induced loss of expression of occludin. Indomethacin also induced apoptosis via activation of caspase-3 and inhibition of p38 MAPK prevented indomethacin-induced activation of caspase-3. Collectively this data suggests that NSAID indomethacin induces gastric epithelial barrier dysfunction via changes in expression and localization of occludin and induction of apoptosis in a p38 MAPK-dependent mechanism.

Thus irrespective of injury model, activation of ClC-2 protected loss of tight junction barrier by preservation of localization of occludin at the tight junctions. The role of MAPK and apoptosis in NSAID-induced epithelial barrier dysfunction is not entirely clear. It is possible that ClC-2 mediated intracellular trafficking of occludin, as demonstrated in recent studies, and role of occludin as signaling molecule in the apoptotic event, as emerged in recent literature forms the basis of ClC-2 mediated epithelial barrier function. Further studies are needed to elucidate molecular mechanisms of ClC-2 mediated epithelial barrier function.
Role of CIC-2 and MAPK in Gastric Barrier Function

by
Meghali Prashant Nighot

A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Comparative Biomedical Science

Raleigh, North Carolina
2012

APPROVED BY:

__________________________  ______________________________
Dr. Anthony T. Blikslager                                          Dr. Sam L. Jones
Chair of Advisory Committee

__________________________  ______________________________
Dr. Jody L. Gookin                                                     Dr. Adam Moeser
BIOGRAPHY

Meghali Thakre Nighot was born and brought up in Nagpur, a city in the central part of India. She completed her Bachelor’s degree in Veterinary Sciences and Master’s in veterinary Parasitology from Nagpur Veterinary College. She worked in Poultry industry and Government of India for few years before joining North Carolina State University for PhD study in August 2008.

Meghali would like to continue work in the research area of veterinary and human gastrointestinal pathophysiology.
ACKNOWLEDGMENTS

I would like to acknowledge and appreciate the enormous support that I received throughout my PhD studies at North Carolina State University. I sincerely thank Dr. Anthony Blikslager for providing me this wonderful opportunity of graduate research in his laboratory. I have always found an accomplished mentor and an amazing person in him. I would also like to thank Dr. Sam Jones, Dr. Jody Gookin, and Dr. Adam Moeser for agreeing to be member of my Graduate committee and their valuable advice throughout my degree.

I would like to express my deep gratitude to my fellow laboratory colleagues Tracy Hill, Karen Young, Liara Gonzalez, Katie Tolbert, Liz Lenon, Amelia Gibson, Beth Overman, Eue Jae Sung, Raphael, and Katie Sheats for their support and help with my research studies.

I thank the staff at the Central Procedures Laboratory and the Laboratory of Animal Resources for their excellent technical advice and support. The services of Laboratory for Advanced Electron and Light Optical Methods, CVM are greatly appreciated. Dr. Troy Ghashghaei helped us with confocal microscopy.

This is also a time to say thanks to colleagues and friends including Susan, Mihika, Arun, Aparna, Sudhakar, Shraddha and others for their help throughout this period.

Above all I would like to thank my husband Prashant and son Rudraksh for their personal support and patience at all times. I am grateful to my family, specially my mother Sarita Thakre, my sisters Varsha and Chandrali for which my mere expression of thanks likewise does not suffice. I would also like to thank my late father, Daryaji Thakre and late Sister Dhanashree for their inspiration.
# TABLE OF CONTENTS

LIST OF TABLES.............................................................................................................. vi
LIST OF FIGURES............................................................................................................ vii

**CHAPTER I. ROLE OF ClC-2 CHLORIDE CHANNEL IN GASTRIC MUCOSA**.............................................. 1
   Introduction............................................................................................................ 2
   ClC-2 structure and organization............................................................................ 2
   ClC-2 regulation.................................................................................................... 5
   ClC-2 function....................................................................................................... 7
   Gastric barrier function.........................................................................................10
   Measurement of gastric barrier function.............................................................. 10
   ClC-2 chloride channel in gastric mucosa............................................................ 11
   Role of ClC-2 chloride channel in gastric barrier function................................. 12
   Conclusion……………………………………………........................................ 14
   References............................................................................................................ 16

**CHAPTER II. GASTROINTESTINAL TIGHT JUNCTIONS AND MAPK SIGNALING PATHWAYS**................. 28
   Introduction..................................................................................................... 29
   Structure and composition of epithelial tight junctions....................................... 30
   Signaling to tight junction..............................................................................….. 31
   Mitogen Activated Protein Kinases (MAPK) signaling pathways ..................... 32
   ERK1/2MAPK..................................................................................................... 34
   JNK MAPK.......................................................................................................... 35
   p38 MAPK........................................................................................................... 36
   Effect of MAPK Kinases on tight junctions..................................................... 38
   Conclusion............................................................................................................ 40
   References…........................................................................................................ 42

**CHAPTER III. GASTROPROTECTIVE PROPERTIES OF THE NOVEL PROSTONE SPI-0811 AGAINST ACID-INJURED PORCINE MUCOSA**............................................. 50
   Abstract.................................................................................................................51
   Introduction........................................................................................................... 52
   Materials and Methods....................................................................................... 54
   Results.................................................................................................................. 59
   Discussion............................................................................................................ 63
   Figure legends...................................................................................................... 67
   References............................................................................................................ 75
LIST OF TABLES

Table 1. MAPK signaling at Tight Junction...............................................................39
LIST OF FIGURES

CHAPTER I

Figure 1. Localization of ClC-2 in Parietal cells................................. 15

CHAPTER II

Figure 1. Mitogen Activated Protein Kinases Family......................... 33

CHAPTER III

Figure 1. Electrical responses and $^3$H-mannitol fluxes of acid-injured porcine gastric mucosa.......................................................... 67

Figure 2. Histological and Immunofluorescence findings in acid-injured gastric mucosal tissues............................................................... 69

Figure 3. Western analysis for Expression of TJ proteins in membrane fractions in acid injured porcine gastric mucosa........................................ 71

Figure 4. Electrical responses and $^3$H-mannitol fluxes of acid-injured porcine gastric mucosa with inhibitors................................................. 72

Figure 5. Western analysis for Expression of ClC-2 protein in porcine mucosa......................................................................................... 74

CHAPTER IV

Figure 1. Dose curve for indomethacin-induced loss of TER in MKN-28 cell line..................................................................................... 101

Figure 2. Effect of Cobiprostone (SPI-0811) on indomethacin-induced decrease in TER and increase in epithelial permeability in MKN-28 cells........... 102

Figure 3. Western expression of TJ proteins........................................... 103

Figure 4. Immunolocalization of TJ proteins.......................................... 104

Figure 5. Expression of activated caspase-3....................................... 105
Figure 6. Western analysis for Expression of ClC-2 in MKN-28 cells………..106

Figure 7. Effect of Cobiprostone (SPI-0811) on indomethacin-induced decrease in the transepithelial resistance in stomach mucosa…………………………...107

CHAPTER V

Figure 1. Dose curve for indomethacin-induced loss of TER in MKN-28 cell line………………………………………………………………………… 132

Figure 2. Effect of MAPK inhibition on indomethacin-induced changes in the epithelial barrier in MKN-28 cells……………………………………….. 133

Figure 3. Western analysis of Tight Junction proteins………………………… 134

Figure 4. Western analysis of Phosphorylation of MAPK………………….. 135

Figure 5. Immunolocalization of TJ protein occludin ………………………… 136

Figure 6. Expression of activated caspase-3……………………………….. 137

Figure 7. Effect of MAPK inhibition on indomethacin-induced alterations in the gastric barrier function………………………………………………...... 139
CHAPTER I

LITERATURE REVIEW

ROLE OF CIC-2 CHLORIDE CHANNEL IN GASTRIC MUCOSA
**Introduction**

The ClC super family of voltage-gated Cl⁻ channels consists of nine isoforms that are expressed in a wide variety of tissues and organs. The mutation/disruption of some of the ClC isoforms is associated with a variety of human or animal diseases. One of these isoforms, ClC-2, has been shown to have a critical functional role in tissues such as brain, testis, retina, and intestine. The physiological function and regulation of ClC-2 channels in the intestine has been intensely researched as a substitute for dysfunctional CFTR in the fatal condition of cystic fibrosis. The work in the last two decades has demonstrated a variety of tissue specific functions of ClC-2 besides chloride transport activity. This paper will review the latest knowledge about function and regulation of ClC-2 in general, and specifically its role in the gastric mucosa.

**ClC-2: Structure and distribution**

Based on their localization and function, the nine ClC genes identified so far in mammals can be categorized into 3 groups: isoforms that reside in the plasma membranes and are involved in membrane ion transport and membrane excitability (ClC-1, -2, -Ka/K1, and -Kb/K2); channels that are expressed primarily in the intracellular endosomal-lysosomal system (ClC-3, -4, and -5), and channels that are present in intracellular membranes and may regulate luminal chloride concentration in the intracellular organelles and vesicles (ClC-6 and -7) (57). Although the basic feature of ClC channels is voltage dependent gating with either inward or outward rectification of Cl⁻ concentrations, isoforms such as ClC-4 or ClC-5 which resides in the intracellular vesicles are Cl⁻/H⁺-exchangers (35). The overall importance of
ClC members in tissue homeostasis has been demonstrated by, mouse models, experimental expression systems and human diseases associated with either mutations or functional defects in ClC family members e.g. myotonia (ClC-1) (41), epilepsy (ClC-2) (35), Bartter III- renal salt loss (ClC-Kb) (25), Dent’s disease- proteinuria (ClC-5) (63), and osteopetrosis (ClC-7) (42). The crystal structure of the ClC identified by Dutzler et al. (23) in Salmonella enterica and Escherichia coli aided greatly in defining its function and regulation. The membrane spanning 18 helices form two halves of the double barreled structure and two identical pores. A glutamate residue that protrudes into the pore is proposed to be a binding site for chloride (36). The gating mechanism of ClC-2 is thought to be influenced by intracellular Cl\(^{-}\) concentrations. According to this hypothesis, E217, the conserved glutamate residue, acts as a hyper polarization-dependent protopore gate in ClC-2 and that access of intracellular Cl\(^{-}\) to this site stabilizes the open state of ClC-2 channel (53). Two large highly conserved CBS (cystathionine beta synthase) domains in the C terminus of mammalian but not bacterial ClC-2 have been a subject of interest to researchers. In vitro studies have shown that the CBS domains from several proteins, including ClC-2, can bind to nucleotides like ATP suggesting that they may act as energy sensors (66).

A combination of classical techniques of electrophysiology and cell and molecular biology have been used to demonstrate the presence of ClC-2 mRNA in corneal endothelium and stroma (20), cultured human aortic and coronary vascular smooth muscle cells, and aortic endothelial cells (43), human leukemic cell lines (T, B, and myeloid type), T cells, B cells and neutrophils (37), and sarcolemmal membranes of atrial and ventricular walls in cardiac tissue (9). In neural tissue, ClC-2 was localized in the membrane of dendrites,
dendritic spines, cell bodies and axon initial segments of neurons, and in the end feet of astrocytes. The presence of ClC-2 in axonal membranes with intense γ-aminobutyric acid (GABA) responsive synaptic transmission activity has been demonstrated to be consistent with chloride transport that leads to efficient GABA (A) receptor-mediated inhibition of synaptic transmission (68, 72).

For years, the role of ClC-2 in the respiratory tract and gastrointestinal tract was pursued as a rescue channel to ameliorate cystic fibrosis; a condition caused by defective function of another chloride channel, Cystic Fibrosis Transmembrane Receptor (CFTR). Consistent with this notion, localization and expression of ClC-2 in the apical membrane of airway epithelium was shown to be regulated in a developmental fashion (50, 70). The reports on expression of ClC-2 in the stomach, however, are not conclusive, and are discussed in detail later in this review. The expression of ClC-2 is reported in several studies, but there are some disparities particularly related to its expression in different sections of the intestine. In a group of studies, ClC-2 was detected either in the cytoplasm in a supranuclear compartment or in the basolateral membrane of human, and murine and guinea pig colon cells (12, 44, 62). ClC-2 was found to be localized to the apical membrane adjacent to the apical tight junction complex in murine small intestine (29) but on the basolateral membrane in the duodenum (62). In the human intestinal Caco-2 cell line, expression of ClC-2 was shown to be limited to the apical membrane in one study (49), and to the basolateral membranes in another study (62). A recent report describes abundant presence of ClC-2 on the basolateral membrane in the early distal colon compared to smaller amount of ClC-2 present in the late distal colon (13).
ClC-2 regulation

Regulation of ClC-2 has been described in terms of transcriptional regulation, biochemical modification by phosphorylation, and intracellular trafficking that modulates its abundance in the plasma membrane. In airway epithelium, expression of ClC-2 has been shown to be regulated by the transcriptional factors SP1 and SP3, particularly during the perinatal period (15, 32). Furthermore, expression of ClC-2 in fetal (preII-19) and adult (L2) rat lung Type 2 cell lines as well in the young murine lung correlated well with phosphorylation and glycosylation of SP1 (71). Single amino acid polymorphs in the cytoplasmic region of the ClC-2 gene (CLCN2) have been shown to affect the surface expression and voltage- or cell-swelling-stimulated channel gating (61). In addition, exogenously expressed ClC-2 transcripts in the Calu-3 lung adenocarcinoma cell line were stabilized by IFN-gamma (15). The abundance of ClC-2 in the plasma membrane is also modulated by membrane composition. For example, the abundance of ClC-2 in detergent soluble fractions and its activation kinetics were increased by cholesterol depletion and oxidative stress in the HEK293 cell model (30). Aside from reduction of the abundance of ClC-2 in the plasma membrane after cholesterol repletion, the modulation of ClC-2 gating was less cholesterol dependent in the tissues that express ClC-2 endogenously. Thus, the presence of ClC-2 in the lipid raft of the cell membrane is critical for its interaction with other proteins in the membrane, its trafficking within the cells, and also its functional modulation (17). The N-terminus of ClC-2 was shown to be capable of binding with actin, and disruption of actin via ATP depletion reduced endocytosis of ClC-2 (1, 21, 22). ClC-2 has also been shown to be associated with heat shock protein Hsp90; this association resulted in increased abundance of
ClC-2 on the cell surface and increased sensitivity of ClC-2 gating towards intracellular chloride concentrations (31). Importantly, the partnership of Hsp90 with ClC-2 would also explain varying expression and activity of ClC-2 in the different expression systems and tissues (16).

Several reports have demonstrated regulation of ClC-2 by different protein kinases. Protein kinase A but not Protein kinase C and Ca++/calmodulin-dependent protein kinase II phosphorylated rat ClC-2 that was exogenously expressed in the TSA-210 cell line; but this phosphorylation did not affect channel activity (60). However, endogenous ClC-2 in colonic T84 cells was a target of Protein Kinase C dependent tyrosine phosphorylation initiated by EGFR signaling (5). At low concentrations, TGF-alpha irreversibly inhibited ClC-2 current by EGFR tyrosine kinase activity dependent on activated phosphoinositide 3-kinase, and protein kinase C. At higher concentrations, TGF-alpha lead to reversible activation of the ClC-2 current, possibly related to intracellular alkalinization (5). In other studies, ClC-2 has been shown to be a target of M phase specific p34(cdc2)/cyclin B kinases and serum and glucocorticoid inducible kinase isoforms SGK1-3 (26, 59, 76). Though the functional relevance of phosphorylation-dependent regulation of ClC-2 is not clear, such regulation appears to be highly dependent on changes in the pH and volume. Among hormonal regulation, estrogen was shown to increase ClC-2 mRNA and protein expression in the rat kidney (51, 58). Expression of ClC-2 in the kidney was also shown to be decreased in hypothyroid rats and increased in hyperthyroid rats. In addition, thyroxine treatment of primary cultured renal proximal tubule cells showed dose dependent increases in ClC-2 mRNA, indicating a possible role of thyroid hormone in ClC-2 regulation (65).
**ClC-2 functions**

Conventionally, ClC-2 has been considered to be a ubiquitous, inwardly rectifying plasma membrane Cl⁻ channel that is activated by membrane hyperpolarization, cell swelling, and mildly acidic extracellular pH (28, 35). However, many of the physiological functions of ClC-2 were derived from knock out (KO) studies or studies in heterogeneous expression systems, and the results from genetic knock studies were not always consistent. Recent studies using newer research tools have been useful in defining the functional role of ClC-2. For instance, contrary to the previous notion that ClC-2 helps maintain synaptic inhibition by lowering intracellular Cl⁻ concentrations ([Cl⁻](i)), new simulation studies show that ClC-2 mediates chloride influx instead of efflux (64). Similarly, regarding the fundamental function of a chloride channel, although ClC-2 has been shown to contribute to native chloride secretion and antisense treatment reduced Cl⁻ secretion in the human intestinal Caco-2 cells (49), additional disruption of ClC-2 in CFTR KO mice did not exacerbate the phenotype (75).

The current literature has different opinions about the function of ClC-2 depending upon its localization. For instance, a very recent study shows that the basolateral ClC-2 channels are required for colonic electroneutral absorption of NaCl and KCl (13) while previous studies showing the apical localization suggest Cl⁻ secretory function. It should also be noted that despite the proposed role of ClC-2 in airways, and gastric secretion, the ClC-2 KO mice do not have abnormalities in lung development and gastric function (8, 52). Interestingly, KO mouse studies have demonstrated an important role of ClC-2 in tissue
homeostasis. ClC-2 knockout mice suffer from severe retinal degeneration and testicular degeneration; both of these tissues have a blood barrier and depend on the supporting tissue for nutrition (retinal pigment epithelial cells and Sertoli cells, respectively) (8, 35, 69). Overall, most of the examples from KO studies suggest an important role of ClC-2 in cell-to-cell contact, communication, and homeostasis aside from chloride transport. ClC-2 was believed to contribute to the regulatory volume decrease in human glioma cells and inhibition of ClC-2 with siRNA was shown to inhibit cell proliferation of a human glioma cell line U-87 (24, 74). Another member of the ClC family, ClC-3, has been shown to mediate swelling-activated Cl⁻ current and cell volume regulation in human gastric epithelial cells (38). The role of ClC family members in tissue homeostasis is also evident from a report where expression of chloride intracellular channel 1 (CLIC1) was found to be significantly (1.95-fold) up-regulated in 67.9% of gastric patients and was well correlated with lymph node metastasis, lymphatic invasion, perineural invasion, pathological staging, and a poor prognosis (14).

Localization of ClC-2 at the apical tight junctions in the intestine has created a lot of research interest. Our laboratory has demonstrated a critical role of ClC-2 in intestinal barrier recovery after ischemic injury. In a chloride secretion and prostaglandin-mediated post-ischemic barrier recovery model in porcine intestine, pharmacological inhibition of ClC-2, but not CFTR, impaired recovery of barrier function (7, 47). Consistent with the localization of ClC-2 in murine small intestine, porcine intestine also revealed ClC-2 localization on the apical membrane adjacent to the tight junction (47). In subsequent studies, ClC-2 knockout mice were shown to have impaired post-ischemic barrier recovery that is attributable to the
failure of tight junction recovery (56). Although the ClC-2 channels localized at the apical tight junctions could have an advantage in influencing paracellular permeability via chloride transport through paracellular spaces (40), its localization at the tight junction suggests the possibility of its interaction with signaling molecules densely present in this region as well as the tight junction proteins. Indeed, intestinal villous morphology, the structure of tight junctions and paracellular permeability is altered in the absence of ClC-2 (55). Furthermore, ClC-2 mediated intracellular trafficking of the TJ protein occludin appears to be the basis for altered TJ barrier in the absence of ClC-2 (54). Another interesting finding was that lubiprostone, a ClC-2 agonist, improved recovery of barrier function by a mechanism that was not fully dependent on Cl⁻ secretion but associated with tight junction assembly (48). Lubiprostone, which is an approved treatment for relieving idiopathic chronic constipation, has been shown to stimulate chloride transport in T84 cells, and activate currents of exogenously expressed ClC-2 in HEK-293 cell lines (19). Lubiprostone, has also been shown to induce contraction of rat and human stomach longitudinal muscles, via prostaglandin EP(1) receptor (6). In the absence of the ability of lubiprostone to invade gut mucosa (4) and the presence of prostaglandin receptors on the basolateral side of epithelial cells, such an action of this drug is suggested to be mediated via more accessible tissue such as nerve endings that are projected into the gut lumen. In another clinical study, lubiprostone accelerated small bowel and colonic transit, increased fasting gastric volume, and reduced gastric emptying in healthy volunteers (10).
**Gastric barrier function**

Considering the critical role of ClC-2 in intestinal barrier function, we have investigated if ClC-2 plays a role in the gastric barrier function. The gastric barrier is composed of a single layer of columnar epithelium, mucus, bicarbonate layer, and intraepithelial tight junctions residing at the apical-most region of the paracellular space. The gastric barrier serves as the first line of defense against a hostile luminal environment (3). The mucus bicarbonate barrier is the only pre epithelial barrier between the lumen and the epithelium (2). When it is overwhelmed or breaks down in disease, the next series of protective mechanisms come into play, including intracellular neutralization of acid, rapid epithelial repair, and maintenance and distribution of mucosal blood flow. There are two components of this innate mucosal defense: mechanisms that reduce the ability of pathogens and their toxins to invade the mucosa, and mechanisms that ensure rapid repair of defects in the epithelial monolayer (3). The next line of mucosal defense is formed by a continuous layer of surface epithelial cells which secrete mucus and bicarbonate and generate PGs, heat shock proteins, TFFs, and cathelicidins. Because of the presence of phospholipids on their surfaces, these cells are hydrophobic, repelling acid- and water-soluble damaging agents. Interconnected by tight junctions, the surface epithelial cells form a “barrier” preventing back diffusion of acid and pepsin. Once the epithelial barrier is disrupted, epithelial repair mechanisms must rapidly re-form a continuous epithelial monolayer in order to prevent entry of protons and bacteria (3, 34). The remarkable phenomenon of epithelial restitution during which epithelium re-seals mucosal defects in the presence of acidic environment has been a subject of several studies.
Measurement of gastric epithelial barrier function in the *in vitro* and *in vivo* injury models

In order to study mechanisms of CIC-2 mediated gastric epithelial barrier function, it is necessary to be able to sensitively and dynamically measure gastric barrier function. The most sensitive measure of mucosal barrier function is transepithelial resistance (TER), since this measurement reflects the degree to which ions traverse tissue. There are two routes ions may traverse epithelium: transcellular and paracellular. The properties of the paracellular space differ dramatically between distinct tissues based on differences in tight junction structure. The measurements of TER largely reflect the tight junction resistance. Alternatively, the paracellular pathway can be assessed using a variety of probes that selectively traverse epithelium via the paracellular space. An example of such a probe is mannitol, which has been used in permeability studies as mucosal-to-serosal fluxes in tissues placed in Ussing chambers. This approach is also extended to study *in vivo* responses of modulators of intestinal secretion. In the *in vitro* studies, we have also used FITC-Dextran to measure permeability in the MKN-28 cells.

**CIC-2 chloride channel in the gastric mucosa**

In the stomach, protons are actively secreted by $H^+K^+$-ATPase that is present in the apical membrane of gastric parietal cells. However, it has not been established which channel contributes to apical $Cl^-$ transport required for hydrochloric acid (HCl) secretion. Concerning the involvement of CIC-2 in gastric acid secretion, previous reports in the literature have been divided and controversial. One group has reported that CIC-2 is the apical $Cl^-$ channel
that may be involved in gastric acid secretion based on the following results: (1) CIC-2 was cloned from rabbit gastric mucosa and its electrophysiological properties including anion selectivity ($\Gamma^- < Cl^-$) were similar to those of the native channel in H$^+$,K$^+$-ATPase-containing gastric vesicles (18, 19); (2) CIC-2 in rabbit (45, 46) and human gastric mucosa (19) is activated by cAMP-dependent phosphorylation, and this mechanism is consistent with an essential role of intracellular cAMP in the HCl secretory mechanism; and (3) anti-CIC-2 antibody showed signals in the rabbit gastric parietal cells (67). In contrast, other groups have suggested that CIC-2 is not associated with gastric acid secretion based on the following results: (1) histamine-stimulated gastric acidification in CIC-2-deficient mice was not significantly different from that in wild-type mice (33); (2) anion selectivity of CIC-2 cloned from rats and rabbits is $Cl^- < \Gamma^-$ (11, 39); (3) CIC-2 in rats (33, 67) and rabbits (67) cannot be activated by cAMP-dependent phosphorylation. Clearly, further research is required to identify the precise function of CIC-2 in Cl$^-$ secretion in gastric HCl secretion.

**Role of CIC-2 chloride channel in gastric epithelial barrier function**

The gastric mucosa is continuously exposed to both physical and chemical insults due to dietary contents and digestive process itself. Other insults include the use of non-steroidal drugs that in the presence of high luminal hydrogen ion and pepsin concentrations lead to severe injury (27). Recently, *H. pylori* have also been shown to cause gastric epithelial barrier dysfunction through dislocation of TJ protein occludin (73). Despite multiple types of insults, the gastric mucosa withstands these harmful agents because of the buffering system...
known as the mucosal barrier. Failure of this defense leads to lesions ranging from the single mucosal cell wound to the progression of ulceration and penetration into the mucosa.

In our experimental studies we have found that CIC-2 agonist (Cobiprostone, Sucampo pharmaceuticals Inc.) protected gastric injury, both, \textit{ex-vivo} and \textit{in vitro}. Our data demonstrate that CIC-2 agonist prevented gastric epithelial barrier dysfunction in porcine model of acid-injury and NSAID-induced injury model in the gastric cell line. Furthermore, in our studies CIC-2 activation protected gastric mucosal barrier dysfunction via preservation of expression and localization of tight junction protein occludin. In the human gastric cell line, indomethacin-induced gastric epithelial barrier dysfunction was prevented by CIC-2 agonist by maintaining TJ barrier via preserving expression of occludin as well via preventing indomethacin-induced apoptosis. The molecular mechanism of protective role of CIC-2 against gastric epithelial barrier dysfunction and apoptosis needs to further investigated.

In other set of studies we focused on the role of the CIC-2 in gastric gland organization. Our studies utilizing genetically modified mice suggest that CIC-2 could be a candidate in acid secretion, and absence of CIC-2 causes disorganization of gastric mucosa. We have found that lack of CIC-2 lead to disorganization of the gastric gland and affected various cell populations with the gastric pit. Especially, parietal cell population and expression of H⁺K⁺ATPase was reduced affecting the acid secretion in the CIC-2 knockout mice. We also confirmed the localization of CIC-2 in the parietal cells. Thus our data from these studies indicate that CIC-2 is required for proper differentiation, function, and
organization of gastric glands (Figure 1). Further studies are needed to elucidate the role of ClC-2 in the development and organization of gastric mucosa.

**Conclusion**

In spite of research interest in the function of ClC-2 as a rescue chloride secretory channel for a serious genetic disease of Cystic Fibrosis, the studies so far have not established physiological role of ClC-2. However it is clear that ClC-2 plays an important role based on its localization in various types of tissues such as air ways, gastro-intestinal tract, retina, testis, nervous tissue etc. The physiological role and function of ClC-2 also varies within a system. For instance, localization and function of ClC-2 appears to be different within different segments throughout the length of gastrointestinal tract. On the other hand, several studies including those from our laboratory have indicated role of ClC-2 in functions such as proliferation, tissue homeostasis, and particularly paracellular tight junction barrier. Besides the current studies on role of ClC-2 in the gastric mucosa, our laboratory have demonstrated that intestinal villus and tight junction structure is altered in the absence of ClC-2. In the intestinal injury model, presence of ClC-2 was found to be critical for the recovery of tight junction barrier. Further link between ClC-2 and the tight junctions was demonstrated in a cell culture model where ClC-2 was found to regulate intracellular trafficking of tight junction protein occludin in a caveolin-1 dependent manner. The presence of ClC-2 on the membrane, particularly within the lipid raft domain of the plasma membrane is very interesting in terms of its possible interactions with other membrane proteins, and will surely be explored in further research studies.
Figure 1. ClC-2 is localized in the parietal cells and have functional role in secretion, barrier function, and organization of the gastric glands.
REFERENCES


48. Moeser AJ, Nighot PK, Engelke KJ, Ueno R and Blikslager AT. Recovery of mucosal barrier function in ischemic porcine ileum and colon is stimulated by a novel agonist of the


CHAPTER II

LITERATURE REVIEW

GASTROINTESTINAL EPITHELIAL TIGHT JUNCTIONS AND MAPK SIGNALLING PATHWAYS
**Introduction:**

Tight junctions (TJs) form a barrier to the paracellular diffusion of ions and solutes across the epithelia. The role of signaling pathways that regulate organization of TJs and permeability has been a focus of intense research efforts. Recent research indicates that different types of signaling proteins and signaling pathways are associated with tight junction function. Specifically, MAPK which are among the best characterized signaling pathways in terms of the control of cell growth and differentiation, have recently been shown to play an important role in the regulation of TJ and paracellular permeability.

The fundamental function of the epithelial cells is to maintain homeostasis of the internal environment by exchange of substances between the two intraluminal and intracellular compartments. Movement of solutes, ions, and water occurs through both the transcellular and the paracellular pathway via pumps and channels and TJs, respectively. TJs form a paracellular diffusion barrier or a gate that regulates epithelial permeability, and an intramembrane diffusion barrier or a fence, which restricts the apical-basolateral diffusion of membrane components. Although the identification of the molecular mechanisms that regulate TJs are incompletely understood, many recent studies show that tight junction proteins are important components of various signaling pathways that regulate critical processes such as epithelial barrier function, cell growth, cell differentiation and cell death.
**Structure and composition of epithelial tight junctions**

TJs consist of a belt-like network of complex strands that define the boundary between the apical and basolateral membrane of epithelial cells. The prevalent model of TJs is a structure composed of a stable multi protein complex composed of integral and peripheral membrane proteins (2, 37). TJs regulate diffusion of ions and small hydrophilic molecules through the paracellular space. Epithelial tight junctions are composed of three main transmembrane proteins – occludin, claudins and junction adhesion molecules (JAMs), and cytoplasmic plaque protein such as zonula occludens-1, 2 and 3 (ZO-1,2,3). The transmembrane proteins mediate cell adhesion and paracellular diffusion barriers, whereas the cytoplasmic plaque proteins such as ZO-1 are a type of “adaptor” proteins which are interposed between the transmembrane proteins and the cytoskeleton. Although the physiological relevance of cytoskeletal interactions with the TJ has not been fully determined, most of the evidence indicates that signaling to the actin cytoskeleton is of fundamental importance to the regulation of TJ assembly and function. TJ proteins are important components of numerous signaling pathways that regulate various cellular processes. These proteins can either stimulate or repress expression of particular genes involved in signaling pathways. Conversely, the intracellular signaling pathways can regulate the molecular composition and permeability of TJs. Of particular relevance to gastric epithelial physiology, gene expression studies have revealed that *CLDN18* is expressed exclusively in gastric cells while high levels of *CLDN3, CLDN4, and CLDN7* are present in the neoplasm originating from gastric cells (23).
Signaling to tight junction

The intracellular signaling pathways that regulate TJ assembly and function have received a lot of interest recently, but it is often difficult to differentiate between the pathways that regulate tight junctions and those that alter paracellular permeability. In other words, regulation of TJ assembly and paracellular permeability are to some extent different domains where signaling pathways can act selectively or have overlapping effects. Several different types of signaling pathways and proteins have been shown to participate in the stimulation of TJ assembly (5, 8). It has been shown that PKA (Protein Kinase A) activation prevents the disassembly of tight junctions in a calcium switch model (11, 14, 31); however its role in TJ permeability is controversial. Also, G-coupled Proteins have been shown to regulate TJ assembly (19, 30). The G protein-mediated TJ regulation is explained by increased paracellular permeability caused by the action of Ga12 on ZO-1 (20, 36). Similarly, it has also been demonstrated that Protein Kinase C is involved in both assembly and disassembly of TJs. Inhibition of PKC blocked both TJ assembly and disassembly, indicating the dynamic nature of transient activation of PKC in TJ regulation (5, 7, 16, 25, 30). Similar experiments have also suggested that Rho family proteins are linked to both the regulation of junctional assembly of AJs and TJs (32, 36). Bruewer et al., (12) based on mutagenesis study, demonstrated that Rho family GTPases such as RhoA, Rac1, and Cdc42 regulate epithelial intercellular junctions via distinct morphological and biochemical mechanisms that correlate to any imbalance in active/resting GTPase levels. Emerging evidence has shown that MAPK signaling is also
important in neoplastic processes involving gastric mucosa. For instance, claudin-18a2 is a splice variant of claudin-18 gene that is involved in cell adhesion as well as the paracellular barrier for H⁺ in the gastric mucosa (22). In gastric cancers of intestinal phenotype claudin-18a2 is down regulated and activation of PKC with phorbol 12-myristate 13-acetate (PMA) increases claudin-18a2 expression in MKN45 human gastric cancer cell line (41). Furthermore, the expression of claudin-18a2 for which two activator protein (AP)-1 promoter binding sites were identified to be critical, was suppressed by the use of PKC and MAPK inhibitors, indicating importance of PKC/MAPK/AP-1 signaling pathway in the regulation of TJ protein claudin-18 (41). Recent evidence indicates that besides the well-established role of MAPK signaling in cell differentiation and cell proliferation, MAPK are also involved in the regulation of epithelial barrier dysfunction via TJ permeability (1, 18, 21, 33, 34).

Mitogen Activated Protein Kinase (MAPK) signaling

Mitogen activated protein kinase (MAPK) cascades are among the best characterized intracellular signaling pathways. This cascade mainly consists of three–kinase module comprising of MAPK Kinases Kinases (MAPKKK) that activates a MAPK Kinase (MAPKK) that in turn activates MAPK. The activated MAPK then translocates to the nucleus where they phosphorylate and activate various transcription factors and thereby modulate gene expression. To date, six distinct groups of MAPK have been identified in mammalian cells, including extracellular signaling regulated 1 and 2 (ERK1/2), p38 (p38α, p38β, p38γ, and p38δ), and c-Jun NH2-terminal Kinases (JNK1, JNK-2, JNK-3)
(14, 18). Extracellular kinases that have been added more recently included ERK3, ERK4, ERK5, ERK 7, and ERK 8 (7, 25). The most well characterized groups of MAPK are ERK1/2, p38, and JNK (Figure 1) which are activated by either extracellular signals or by various stress factors.

Figure 1: Mitogen-activated protein kinases (MAPK) are a family of Ser/Thr protein kinases widely conserved among eukaryotes and are involved in many cellular programs such as cell proliferation, cell differentiation, cell movement and cell death. MAPK signaling cascades are organized hierarchically into three-tiered modules. MAPKs are phosphorylated and activated by MAPK-kinases (MAPKKs), which in turn are phosphorylated and activated by MAPKK-kinases (MAPKKKs). The MAPKKKs are in turn activated by interaction with the family of small GTPases and/or other protein kinases, connecting the MAPK module to cell surface receptors or external stimuli (adapted from http://www.cellsignal.com/reference/pathway/MAPK_Cascades.html)
**ERK1/2**

One of the MAPK cascade modules is the ERK signaling pathway, also known as Extracellular Signaling MAPK pathway. ERK1/2 is activated by MEK1/2 which in turn is activated by Raf isoform that is activated by GTPase, and Ras whose activation is dependent on Tyrosine kinases. EGF receptor activation also turns on ERK pathway. ERK has been recently shown to play role in modulating TJ permeability. Some studies have shown that activation of MAPK by active Raf-1 in salivary glands and active Ras in MDCK cells disrupts the TJ (26-28). Consequently treatment of Ras transformed MDCK cells with a MEK inhibitor results in stabilized expression of occludin and zo-1 (14). Disruption of TJ by Ras is associated with a decrease in the level of occludin (1, 9) whereas Raf-1 decreases the expression of claudin-1 (27, 39). In intestinal Caco-2 cells, ERK was shown to directly interact with the C-terminal tail of occludin, and this interaction mediated prevention of H₂O₂-induced TJ barrier disruption (10). In recent studies in MDCK cells EGF induced ERK1/2 and increased TJ permeability by up regulation of claudin-2 and down regulation of claudin-4 (21). Recently Aggarwal et al. (1) demonstrated that the role of ERK activation in the regulation of TJ depends upon the tissue being studied; in some cells it disrupts the junctions while in other it prevents disruption, based on differences in its subcellular distribution and ability to regulate the association of PKCe and PP2A with the tight junctions. MAPK signaling is also important in gastric inflammation and inflammation-associated carcinogenesis. For example, Cytotoxin-associated antigen (CagA), a *H. pylori* immunoprotein can transform gastric epithelial cells through activation of the ERK1/2 pathway (43). Clinically, CagA
has been shown to increase gastric cancer risk via activation of the ERK signaling pathway and ERK single-nucleotide polymorphism (SNP) rs5999749 was found to be one of the important genetic determinants for gastric carcinogenesis (40). Although the mechanism by which CagA-mediated ERK signaling affects TJ barrier in the stomach mucosa is not clear, intracellular CagA is associated with the epithelial TJ intracytoplasmic protein ZO-1 (38). Furthermore, the interaction of CagA with PAR1b/MARK2 polarity-regulating kinase has been shown to disrupt the tight junctions (3).

**JNK-c-Jun-NH2 terminal activated Protein Kinases (SAPK- Stress activated kinases)**

JNK are activated by a wide array of environmental stressors and are commonly referred to as stress activated protein Kinases. In human cells, the JNK protein subfamily is created by differential splicing of three genes, JNK1, JNK2, and JNK3; JNK3 expression is restricted to the brain (18, 29). Recent studies have demonstrated a role of JNK in the TJ integrity in epithelial cells (34). According to Naydenov et al. (29), JNK plays an important role in F-actin remodeling and disassembly in epithelial junctions, and JNK-mediated phosphorylation and activation of ERM proteins may contribute to the cytoskeleton remodeling. Another study by Carrozzino et al. (13) found that in MDCK and mammary epithelial cell lines, inhibition of JNK with SP600125 inhibited the increase in the permeability and expression of claudin-4 and claudin-9 but down regulated expression of claudin-8 suggesting that specific tight junction proteins are
regulated by different MAPK components. Similar recent studies by Samak et al. (34) used calcium switch models in Caco-2 cells and osmotic stress in JNK1 and JNK 2 knock out mouse studies to demonstrate that JNK-2 and not JNK-1 is involved in inducing epithelial barrier dysfunction via down regulation of the TJ protein ZO-1 and up regulation of claudin-4 following osmotic stress-induced TJ disruption in intestinal epithelium. Further in vivo studies are needed to elucidate the exact roles of distinct isoforms of JNK and the mechanism involved in TJ disassembly.

**p38 Mitogen Activated Protein Kinases**

The p38 mitogen activated proteins kinases (p38) are stress-activated kinases that, together with ERK1/2, and JNK form the critical components of the mitogen activated protein kinase (MAPK) cascades. Four p38 isoforms (p38α, p38β, p38γ, and p38δ) have been identified so far. This pathway in general is inhibited pharmacologically by SB203580, a pyridinyl molecule. Recently several studies have shown tight junction disruption via phosphorylation of p38 in various cell models. Initial studies in the MKN28 gastric cell line have shown that aspirin activates all three MAPK kinase pathways, ERK, JNK and p38 but epithelial barrier dysfunction caused by aspirin in these cells occurs through phosphorylation of p38 and down regulation of claudin-7 (33). Similarly, basal inhibition of p38 isoforms in MDCK and mammary cell lines targeted different tight junction proteins and increased epithelial permeability (13). According to this study, silencing the p38α isoform specifically increases claudin-4 and claudin-8 mRNA while silencing p38β affected claudin-4 mRNA, demonstrating isoform specific
effects of p38 on claudin expression. Recently Chuenkitivanon et al. (15) showed that H$_2$O$_2$ induced hyper permeability in ECV304 endothelial cells originating from human umbilical vein involved phosphorylation of p38 MAPK and down regulation of ZO-1 and occludin. It is presumed that p38 MAPK-mediated disruption of tight junctions and adherens junctions contributes to acquisition of malignant properties noted in poorly differentiated gastric phenotypes. In one study, expression of E-cadherin, ZO-1, occludin and claudin-4 was found to be reduced in poorly differentiated gastric cancer cell lines KATO-III and MKN45 cells as compared to well differentiated MKN7 and MKN28 gastric cells (4).

In our experiments, we have found that all MAPKs were phosphorylated following indomethacin-induced injury but there was only a significant increase in phosphorylation of p38 MAPK and JNK as determined by western analysis and densitometry ($p<0.05$). The present study shows that even though indomethacin phosphorylates p38, JNK, and ERK MAPK, indomethacin-induced TER and increased permeability was only attenuated by inhibition of p38 and JNK MAPK. We also found that occludin expression and localization was preserved by inhibition of p38 and not JNK. Though it appears that JNK MAPK was involved in indomethacin-induced loss of TER, its specific action on TJ was not studied. It is quite possible that, as reported previously (13), JNK mediates TJ barrier via specific claudin isoform not examined in this study. Our current data support the idea that disruption of barrier function involves MAPK activation and specific tight junction protein is targeted by particular MAPK component. Thus p38 MAPK could be a potential therapeutic target in preventing indomethacin induced damage to the gastric mucosa.
Effect of MAPK on Tight Junctions

Recent studies on TJs and MAPK clearly indicate the role of MAPK signaling in epithelial barrier dysfunction via induction of TJ assembly or TJ disassembly. It is apparent that all MAPK cascade components are involved in TJ regulation and target different tight junction proteins. The role of MAPK in TJ regulation also seems to be specific for a particular cell line used in experimental studies. The mechanisms involved in specific actions of the three main components of MAPK on specific tight junction proteins in different barrier dysfunction models in varied cell lines is tabulated in table 1. This information pertains to how different stress factors or extracellular signaling leads to activation of specific MAPK components and thus target specific tight junction proteins to alter basal barrier function.
Table 1: MAPK signaling at Tight Junction

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HUVEC</td>
<td>H₂O₂-injury</td>
<td>ERK1/2</td>
<td>occludin</td>
<td>(24)</td>
</tr>
<tr>
<td>2</td>
<td>Caco-2</td>
<td>H₂O₂-injury</td>
<td>ERK</td>
<td>ZO-1, occludin</td>
<td>(10)</td>
</tr>
<tr>
<td>3</td>
<td>MDCK</td>
<td>Calcium ++ switch</td>
<td>ERK</td>
<td>occludin</td>
<td>(6)</td>
</tr>
<tr>
<td>4</td>
<td>MDCK</td>
<td>EGF treatment</td>
<td>ERK1/2</td>
<td>claudin-4(up) claudin-2(dn)</td>
<td>(21)</td>
</tr>
<tr>
<td>5</td>
<td>Rat alveolar cells</td>
<td>Cyclic stretch</td>
<td>JNK</td>
<td>occludin</td>
<td>(17)</td>
</tr>
<tr>
<td>6</td>
<td>T84, SK-CO15</td>
<td>Ca ++ switch</td>
<td>c-JNK</td>
<td>occludin, ZO-1</td>
<td>(29)</td>
</tr>
<tr>
<td>7</td>
<td>Caco-2</td>
<td>Ca ++ switch, osmotic stress</td>
<td>JNK-2</td>
<td>ZO-1, claudin-4</td>
<td>(34)</td>
</tr>
<tr>
<td>8</td>
<td>31EG4-2</td>
<td>Basal inhibition</td>
<td>JNK, p38,</td>
<td>claudin-4, -9, and</td>
<td>(13)</td>
</tr>
<tr>
<td>9</td>
<td>MKN28</td>
<td>Aspirin</td>
<td>p38</td>
<td>claudin-7</td>
<td>(33)</td>
</tr>
<tr>
<td>10</td>
<td>MKN45</td>
<td>PKC activation</td>
<td>MAPK</td>
<td>Claudin-18a2</td>
<td>(41)</td>
</tr>
</tbody>
</table>
Conclusions

TJ are specifically connected to a series of signaling pathways that transduce extracellular information towards the cell interior to modulate cell behavior and response. Recent studies have shown that multiple signaling pathways are linked to these processes, and understanding of these complex mechanisms has just begun. Although the role of MAPK pathways in cell differentiation, proliferation and cell death (apoptosis) are well understood, the role of TJ in cell differentiation and cell death is unclear. However, recent studies indicate that TJs have a central role in processes that regulate epithelial proliferation and differentiation (7, 34, 35). MAPK have so far been shown to induce epithelial barrier dysfunction in gastrointestinal cells in different injury models. Knowing the fact that some of the models, particularly those that focus on NSAID-induced apoptosis via MAPK, it would be interesting to see what role TJs play in the induction of cell death by the NSAIDs. More evidence and studies are required to elucidate if MAPK affects gene expression of particular tight junction proteins or how the composition of the TJ is modulated by MAPK signaling. Formation and assembly of TJs would involve several steps in protein synthesis and degradation, from the regulation of gene transcription and mRNA processing to the regulation of protein turnover by stabilization of mRNA. Isoform specific targeting of claudin proteins by the MAPK pathways in the gastric mucosa is an example of a current exciting research finding. Experimental results, however, need cautious interpretation considering the variation in the expression of claudin isoforms in different cell culture models. In addition, TJ associated signaling systems are probably affected, and influenced by other pathways that control
proliferation, cell differentiation, apoptosis, and epithelial barrier dysfunction. Therefore, it will be important to study cross talk between TJs and other well established regulatory systems such as cadherin–based adhesion signaling and signaling from the extracellular matrix (7, 42). It seems that activation or phosphorylation of MAPK is associated with epithelial barrier dysfunction and thus is of immense therapeutic potential in important gastrointestinal disorders/diseases such as Inflammatory Bowel Syndrome (IBS), Inflammatory Bowel Disease, and NASID induced gastro-enteropathy.
REFERENCES


Chapter III

GASTRO-PROTECTIVE PROPERTIES OF THE NOVEL PROSTONE SPI-0811 IN ACID-INJURED PORCINE GASTRIC MUCOSA

Submitted to World Journal of Gastroenterology
ABSTRACT

Aim: To evaluate the protective properties of novel prostone ClC-2 agonist SPI-0811 in porcine model of gastric acid injury.

Method: Porcine gastric mucosa was mounted in Ussing chambers and injured by bathing mucosal tissues in an HCl Ringer’s solution (pH 1.5) with or without SP1-0811(1µM), CFTR inhibitor (inhibitor 172, 10µM, apical) and ClC-2 inhibitor ZnCl2, 300µM, apical), on the apical surface of tissues. Transepithelial resistance and mucosal-to-serosal ³H-mannitol fluxes were measured over a 90-minute period. Tissues were analyzed by morph metric techniques, immunofluorescence and by western blots.

Results: Compared with control tissues, acid exposure decreased TER and increased ³H-mannitol flux. Pretreatment of gastric mucosa with SPI-0811 was protective against acid-induced decreases in TER (TER Ω.cm² = 50 vs. 100) and abolished increases in flux (³H-mannitol flux 0.10 vs. 0.04 µM.cm²). Evidence of histological damage in the presence of acid was markedly attenuated by SPI-0811. Immunofluorescense and western analysis for occludin revealed enhanced localization to the region of the tight junction after treatment with SPI-0811. Pretreatment with the ClC-2 inhibitor ZnCl2, but not the selective CFTR inhibitor 172, attenuated SPI-0811-mediated mucosal protection, suggesting a role for ClC-2. Prostone may serve both protective and reparative roles in injured tissues.

Conclusion: ClC-2 agonist SPI-0811 stimulated enhancement of mucosal barrier function by protecting tight junction protein occludin in porcine gastric mucosa and thus protected the gastric acid injury in porcine stomach.

Keywords: Stomach, Mucosal permeability, ClC-2 chloride channel, tight junction
INTRODUCTION

It is becoming increasingly evident that many patients suffer from gastric ulcers, particularly in groups of patients such as those in intensive care facilities [1-4]. This suggests that medications that provide gastro protection have the potential to reduce morbidity associated with gastric ulceration. For decades, agents that suppress acid secretion [5-7] have been widely used for treatment of gastric ulcers [1,7-10]. Gastric ulcer disease and repair is complex, involving inflammation, cell proliferation, formation of granulation tissue, and angiogenesis [3,4,11]. However, gastro protection has also been studied in depth. For example, studies with rebamipide or misoprostol, geranylgeranyl hydrochloride (HSP70) have revealed that these compounds have gastro protective properties as well enhancing ulcer healing [2,7,12]. Nonetheless, our understanding of the mechanisms of gastro protection is incomplete and there is the prospect of novel pharmacological agents, aside from antacids, proton pump inhibitors, and prostanoid activators or analogs, which might protect the stomach [2,13].

The gastric barrier is composed of a single layer of columnar epithelium, mucus, bicarbonate layer, and intraepithelial tight junctions residing at the apical-most region of the paracellular space. The gastric barrier serves as the first line of defense against a hostile luminal environment. The mucus bicarbonate barrier is the only pre epithelial barrier between lumen and the epithelium [14,15]. When it is overwhelmed or breaks down in disease, the next series of protective mechanisms come into play, including intracellular neutralization of acid, rapid epithelial repair, and maintenance and distribution of mucosal blood flow. There are two components of this innate mucosal defense: mechanisms that reduce the ability of pathogens and their toxins to invade the mucosa, and the mechanisms
that ensure rapid repair of defects in the epithelial monolayer \[3,4,15-17\]. The next line of mucosal defense is formed by a continuous layer of surface epithelial cells which secrete mucus and bicarbonate and generate PGs, heat shock proteins, TFFs, and cathelicidins. Because of the presence of phospholipids on their surfaces, these cells are hydrophobic, repelling acid- and water-soluble damaging agents. Interconnected by tight junctions, the surface epithelial cells form a “barrier” preventing back diffusion of acid and pepsin. Once the epithelial barrier is disrupted, epithelial repair mechanisms must rapidly re-form a continuous epithelial monolayer in order to prevent entry of protons and bacteria \[3,4,14,15,18\]. The remarkable phenomenon of epithelial restitution during which epithelium re-seals mucosal defects in the presence of acidic environment has been a subject of several studies.

Acid injury is an important component of gastric ulcer disease, despite more recent findings on the role of Helicobacter pylori which also contributes to this troublesome problem. Aside from injuring epithelium directly, acid causes disruption of interepithelial tight junction (TJ) protein complexes thereby increasing epithelial permeability \[10,14,16,19\]. Models assessing the mechanisms of intestinal injury have demonstrated that the critical event defining disruption of barrier function is the loss of TJ architecture and redistribution of TJ proteins such as occludin from the apical region of the inter epithelial space to the cytosol \[10,20-22\]. In our previous work, we have demonstrated a critical role for Cl\(^{-}\) secretion in restoration of intestinal barrier function in ischemic-injured porcine ileum \[21\]. This appears to be attributable to activation of the Cl\(^{-}\) channel CIC-2, which is localized to the TJ \[21\]. More specifically, prior studies have shown that the nonselective secretory agonist PGE\(_2\) triggered rapid recovery of transepithelial electrical resistance (TER) and reduced mucosal-to-serosal
fluxes of $^3$H-mannitol in ischemic-injured intestinal mucus $^{[20, 22-24]}$. Given the previous findings that activation of ClC-2 by the prostone Lubiprostone $^{[22, 25]}$ enhanced recovery of barrier function in ischemic-injured porcine intestine, the present study was performed to evaluate the ability of the novel ClC-2 agonist SPI-0811 to provide gastro protection in acid-injured porcine gastric mucosa.

**MATERIALS AND METHODS**

**Chemicals**
ZnCl$_2$, Bumetanide, $^3$H-mannitol, and CFTR inhibitor 172 were purchased from Sigma-Aldrich (St. Louis, MO). SPI-0811 was provided by Sucampo Pharmaceuticals Inc., Bethesda, MD.

**Experimental Animals**
All studies were approved by the North Carolina State University Institutional Animal Care and Use committee. Yorkshire crossbred pigs of either sex ~10-15kg body weight were housed individually and maintained on a commercial pelleted feed. Pigs were held off feed, but had free access to water, for 12 h before each experiment. Anaesthesia was induced with xylazine (1.5mg/kg, IM) and ketamine (11mg/kg, IM), after which they were euthanized with pentobarbital (20mg/kg, IV). The entire stomach was clamped proximally and distally with Doyen intestinal forceps.
Ussing chamber studies

After harvesting the entire stomach, it was sharply incised at the lesser curvature and washed in porcine Ringer’s (mM: 154 Na⁺, 6.3 K⁺, 137 Cl⁻, 0.3 H₂PO₄, 1.2 Ca²⁺, 0.7 Mg²⁺, 24 HCO₃⁻, pH 7.4) and maintained in oxygenated (95% O₂/5% CO₂) Ringer’s solution. The fundus portion was isolated for Ussing chamber studies. After stripping the mucosa from the seromuscular layer by using blunt scissors, mucosa was mounted in 3.14cm² aperture Ussing chambers. Gastric mucosa from individual pigs was mounted on multiple Ussing chambers and subjected to acid injury and select treatments. Tissues were initially bathed in 10ml porcine Ringer’s on both mucosal and serosal sides. The serosal bathing solution contained 10mM glucose to maintain tissue viability, and this was osmotically balanced on the mucosal side with 10mM mannitol. Indomethacin (5µM) was added on the serosal and mucosal sides of gastric tissues to prevent prostaglandin production. Bathing solutions were oxygenated and maintained at 37°C by water jacketed reservoirs. The spontaneous potential difference (PD) was measured via Ringer’s-agar bridges connected to calomel electrodes, and the PD was short-circuited via Ag-AgCl₂ electrodes using voltage clamps that corrected for fluid resistance to measure short circuit current (Isc). Transepithelial resistance (Ω·cm²) was calculated from the spontaneous PD and Isc. If the spontaneous PD was between -1.0 and +1.0 mV, tissues were current clamped at ±100 µA for 5 s and the PD was recorded. Isc and PD were recorded at 15 min intervals over a 90 minute experimental period.
Experimental Treatments

Once tissues were mounted on Ussing chambers, treatments aimed at inhibiting ClC-2 \((\text{ZnCl}_2, 300\mu\text{M, apical})\), CFTR \((\text{inhibitor 172, 10}\mu\text{M, apical})\), or NKCC1 \((\text{bumetanide, 100}\mu\text{M, basolateral})\) were added. Alternatively, SPI-0811 was administered to the apical surface of tissues \((1\mu\text{M})\) to enhance epithelial Cl\(^{-}\) secretion via ClC-2. Following a 30 min equilibration period, HCl \((1\text{N in Ringer’s})\) was added to the mucosal surface of the tissue to induce acid injury. The pH was monitored with a pH meter \((\text{Hanna Instruments Inc., Ann Arbor, MI, USA})\) and maintained at 1.5 by adding 1N HCl as needed during the experiment.

Mucosal-to-serosal fluxes of $^3\text{H}$-mannitol

To assess mucosal permeability, 0.2$\mu$Ci/ml $^3\text{H}$-mannitol was placed on the mucosal side of tissues after experimental treatments. After a 15 min equilibration period, standards were taken from the mucosal side of each chamber and a 30 min flux period was established by taking 0.5ml samples from the serosal compartments. The presence of $^3\text{H}$ was established by measuring emission in a liquid scintillation counter \((\text{Rack Beta, Perkin Elmer Life and Analytical Sciences, Boston MA, USA})\). Unidirectional mucosal-to-serosal $^3\text{H}$-mannitol fluxes were calculated using a previously established spreadsheet \(^{[17]}\).

Histological analyses

Tissue samples were collected at 0, 30 and 90 minutes during the experimental period and fixed in 10% formalin for histological evaluation. Paraffin embedded samples were sectioned
(5µm) and stained with hematoxylin and eosin and PAS. For each tissue, mucosal epithelial lining and gastric pits (crypts) were identified to assess the damage caused by acid (pH 1.5).

**Immunofluorescence labeling of occludin**

For this procedure, tissues were embedded in optimal cutting temperature (OCT) medium, frozen, and sectioned at 5µm. Tissue sections were blocked with 2% BSA followed by incubation with rabbit anti-occludin polyclonal antibody (1:150, Zymed, San Francisco, CA, USA) overnight at 4°C. Sections were washed with PBS and incubated for 45 min with FITC-conjugated anti-rabbit secondary antibody. Sections were mounted in fluorescent mounting medium, and well-orientated gastric pits were examined with a photomicroscope linked to a digital camera.

**Gel electrophoresis and western blotting**

Following Ussing chamber experiments, gastric mucosal samples were snap frozen and stored at -70°C prior to performing SDS-PAGE. Tissue aliquots were thawed at 4°C and added to chilled lysis buffer, including protease inhibitors (0.5mM Pefabloc, 0.1mM 4-nitrophenyl phosphate, 0.04 mM glycerophosphate, 0.1mM Na₃VO₄, 40µg/ml bestatin, 2µg/ml aprotinin, 0.54µg/ml eupeptic, and 0.7µg/ml pepstatin A) (Sigma-Aldrich Inc., St. Louis, MO, USA) at 4°C. This mixture was homogenized on ice and then centrifuged at 4°C, and the supernatant was saved. Protein analysis of extract aliquots was performed (BCA Protein Assay Kit, Pierce, Rockford, IL, USA). Tissue extracts (amounts equalized by protein concentration) were mixed with an equal volume of 2 × SDS-PAGE sample buffers
and boiled for 4 min. Lysates were loaded on an SDS polyacrylamide gradient gel, and electrophoresis was carried out according to standard protocols. Proteins were transferred to a PVDF membrane (Immobilon®, Millipore, Billerica, MA, USA) by using an electroblotting minitransfer apparatus. Membranes were blocked at room temperature for 2 h in Tris-buffered saline plus 0.05% Tween 20 (TBST) and 5% dry powdered milk, and then incubated overnight in primary antibody at 4°C in rabbit anti-rat ClC-2 (Alpha Diagnostics, San Antonio, TX, USA), rabbit anti-CFTR (Santa Cruz Biotech Inc., CA, USA) or rabbit anti-occludin (Zymed Laboratories Inc., San Francisco, CA, USA). After multiple washings in TBST, membranes were incubated with horseradish peroxidase conjugated secondary antibody, and developed for visualization of protein with luminol enhancer solution (Pierce, Rockford, IL, USA).

For preparation of detergent soluble and detergent insoluble fractions of gastric mucosa, the tissue samples were extracted in lysis buffer (20mM Tris, 5mM MgCl₂, 0.3mM EGTA, 210μg/ml sodium fluoride, 18.5μg/ml sodium orthovanadate, 30mM sodium pyrophosphate, and complete mini Protease inhibitor cocktail tablet (Thermo Fisher Scientific, Rockford, IL. 61105 USA). Following brief centrifugation to remove debris, Triton X-100 soluble and insoluble fractions were collected by incubation and centrifugation (50,000 rpm for 30 min at 4°C) with lysis buffer containing 0.5% Triton X-100 and 0.5% SDS, respectively. The samples were processed through an SDS sample preparation kit (Thermo Fisher Scientific, Rockford, IL. 61105 USA) and a protein assay was performed before proceeding with western blotting.
Statistical analyses

Data were reported as mean ±SE. For Ussing chamber experiments, an experimental number of 6 was used (n=6). Data was analyzed by using an analysis of variance (ANOVA) and t-test using a commercial statistical package (Sigmastat, Systat Software, San Jose, CA, USA). The significance level was set at $p<0.05$.

RESULTS

Acid injury model

To establish a gastric acid injury model, porcine gastric mucosa was mounted on Ussing chambers. After an equilibration period of 30 minutes the mucosal side was subjected to normal Ringer’s solutions (pH 7.4) or HCl Ringer’s (pH 1.5) for up to 180 minutes. Acid-bathed tissues had a significant decline in TER by 90 minutes and a further decline by 180 minutes (approximately 50% and 150% change in TER following 90 and 180 minutes of exposure to HCl, respectively, fig 1A). Histological examination of the gastric mucosa exposed to acid for 90 minutes revealed injury that was limited to mucosal sloughing and partial damage to the gastric pits, resembling acute peptic ulcer disease. Alternatively, 180 minutes exposure to acid caused profound sloughing of the mucosal lining with damage extending into the gastric pits .Thus for further studies, gastric mucosa was exposed to HCl (pH 1.5) for 90 minutes to establish a more clinically relevant level of acid-induced gastric injury (data not shown).
Effects of the ClC-2 agonist SPI-0811 on TER and mucosal-to-serosal $^3$H-mannitol fluxes in acid-injured porcine gastric mucosa

As previously described, porcine gastric mucosa was mounted on Ussing chambers and the mucosal surface was exposed to acid Ringer’s (pH 1.5) for 90 minutes. Mucosa subjected to acid injury had significantly lower TER values when compared to the gastric mucosa bathed in normal Ringer’s, indicating electrophysiological evidence of disruption of epithelial barrier function. Alternatively, pretreatment of the apical side of gastric mucosa with the ClC-2 agonist SPI-0811 (1µM) nullified decreases in TER due to acid injury (fig 1A). Epithelial permeability was assessed by mucosal-to-serosal fluxes of $^3$H-mannitol in control and acid-injured tissues in the presence or absence of apical SPI-0811. In agreement with TER responses, acid-injured tissues exhibited increases in permeability to $^3$H- mannitol as compared to control uninjured tissue. Pretreatment of the apical surface of mucosal tissues SPI-0811 ablated changes in permeability noted in untreated acid-injured tissues (fig 1B).

Histological and Immunofluorescence microscopic findings in acid-injured gastric mucosa

Gastric mucosa from Ussing chambers after 90 minutes in normal Ringer’s revealed intact gastric pits with intact epithelial lining. Alternatively, when the gastric mucosa was exposed to acid (pH 1.5) over a 90 minute period, extensive epithelial sloughing and erosion extending into the glandular region of the gastric mucosa and sub-epithelium was noted. Tissues pretreated with SPI-0811 had far lesser evidence of mucosal injury in response to
acid, although the epithelium appeared to have flattened to maintain the barrier and the gastric pits appeared dilated (fig 2A).

Using PAS staining for mucus we observed that the loss of mucus by acid injury was seen restored by pretreatment of ClC-2 agonist (fig 2B). In further experiments, we performed Immunofluorescence of occludin to assess the gastric mucosal tight junctions (TJ). As compared to apical immunolocalization of occludin in the control uninjured gastric mucosal lining epithelium, acid-injured tissues revealed loss of occludin Immunofluorescence (fig 2C). In contrast, gastric mucosa pretreated with SPI-0811 and subsequently exposed to acid for 90 minutes had evidence of localization of occludin to the TJ.

**Expression of occludin in gastric mucosal tissues**

Since SPI-0811 prevented increases in paracellular permeability in response to acid, based on reduced $^3$H-mannitol fluxes and apical epithelial localization of occludin, we evaluated expression of occludin. Occludin expression was studied in detergent soluble and detergent insoluble gastric mucosal fractions, using $\beta$-actin expression as a loading control. Tissues exposed to normal Ringer’s had expression of multiple bands clustered at 65kD in the detergent insoluble fraction. The finding of several bands for occludin at its expected molecular weight has been attributed to multiple occludin phosphorylation states $^{[26]}$. The presence of occludin solely in the insoluble fraction was interpreted as an indication of this protein’s propensity to localize to the tight junction. In contrast to tissues bathed in normal Ringer’s, there was very little expression of occludin in acid-treated tissues, possibly because
acid injury was severe enough to cause surface epithelium and inter epithelial tight junctions. On the other hand, occludin expression in the detergent insoluble fraction of tissues treated with SPI-0811 appeared similar to tissues treated with Ringer’s solution alone, suggesting this prostone prevented loss of occludin. Cellular expression of occludin as a whole was also seen markedly reduced compared to control tissue and this loss was prevented with pretreatment with SPI-0811(fig. 3A and 3B).

Role of Cl- channels in SPI-0811-mediated protection of TER and barrier permeability in acid-injured gastric mucosa

To explore the potential role of Cl secretion in SPI-0811-associated gastro protection, mucosa was treated with the NKCC1 inhibitor bumetanide (100µM, basolateral) and SPI-0811 (1µM, apical), which blocked the ability of SPI-0811 to reduce acid injury (data not shown). In more targeted studies in tissues pretreated with SPI-0811, tissues were pretreated with pharmacological inhibitors of the apical chloride channels ClC-2 and CFTR. Pretreatment of acid-injured mucosa with the ClC-2 inhibitor ZnCl2 (300 µM, apical) abolished the gastro protective properties of SPI-0811 as determined by change in TER and mannitol fluxes. On the other hand, pretreatment with CFTR inhibitor 172 (10µM, apical) had no effect (fig 4A, B)

Expression of ClC-2 and CFTR in porcine gastric mucosa

The literature is not clear about the expression of ClC-2 and CFTR in the porcine gastric mucosa, aside from a few studies that describe gastric mucosal changes in CFTR mouse mutants [19]. Therefore, western analyses were performed to study the expression of ClC-2 and
CFTR in porcine gastric mucosa. As shown in figure 5, ClC-2 was expressed ~98 kDA in porcine gastric homogenates. ClC-2 expression was observed in porcine gastric mucosa which confirmed the expression of ClC-2 in porcine stomach. Western analyses showed no evidence of CFTR in gastric mucosa, whereas CFTR was clearly present in porcine jejunum as a positive control (data not shown).

**Discussion**

Prior studies have provided evidence for a critical role of ClC-2 in the recovery of mucosal barrier function in ischemic-injured intestine \[^{20,21,27,28}\]. For example, Lubiprostone has been shown to interact with ClC-2 channels and enhance repair of the epithelial barrier after acute injury by ischemia \[^{22,25,29,30}\]. The aim of the present study was to determine if an alternate prostone, SPI-0811, had a protective role against mucosal injury, and we chose to investigate this in the stomach. Acid plays an integral role in gastric mucosal ulceration \[^{1,3,4,8,15,24,31}\]. Considering the physiological presence of acid to aid digestion in the stomach, mechanisms to provide gastro protection against injury in predisposed patients are critical. To date, this has principally included agents that ameliorate acid secretion. However, according to the results of the present study, prostones appear to provide protection in the face of low levels of pH, which would allow for continued acid secretion for normal digestive processes. Acid causes injury to the gastric mucosa in part by disrupting the TJ protein complexes, thereby increasing epithelial permeability. This in turn allows further injury by permitting permeability to luminal acid, ultimately causing erosion and then ulceration as the mucosa becomes progressively disrupted \[^{3,14,24,32}\]. Among the established methods to study gastro
protection and acute gastric injury are the direct application of acid or ethanol to gastric mucosa \[^{[8,31]}\].

The first aim of our study was to produce acute acid injury resembling ulceration in clinical patients in order to study gastro protection. Accordingly, acid Ringer’s solution was applied to the mucosal surface as previously described \[^{[33]}\]. To mimic peptic ulcer disease, the body of the porcine stomach was used in this study. Optimum gastric injury was achieved (based on TER and histological examination) with a pH of 1.5, over a time period of 90 minutes. In each of the experiments, SPI-0811 was applied to the gastric mucosa prior to acid injury on the apical surface. Earlier studies have shown that Lubiprostone activates Cl\(^-\) secretion by a mechanism associated with recruitment of the tight junction occludin in porcine intestine, thereby aiding recovery of barrier function \[^{[9,22,34,35]}\]. Gastric mucosa was pretreated with SPI-0811 prior to induction of acid injury and showed evidence of gastro protection as evidenced by blockade of changes in TER and mannitol fluxes in tissues exposed to acid. In addition, histological changes in response to acid were ameliorated by SPI-0811. Because of the important role of tight junctions in maintenance of barrier function, we also examined the localization of occludin in tissues pretreated with SPI-0811 and exposed to acid. Tissues injured by acid alone appeared to have complete loss of apical epithelial occludin, possibly because of loss of cells due to severe injury, whereas tissues pretreated with SPI-0811 had evidence of apical epithelial occludin similar to that of control tissues. Further studies evaluating detergent soluble and insoluble fractions using western analyses confirmed the loss of occludin in tissues treated with acid. The reason for the loss of occludin, rather than intracellular movement of occludin, is unknown. The simplest
explanation would be that the extensive reduction in pH resulted in loss of tight junction proteins into the lumen, followed by erosion of epithelium and ultimately ulceration. Tissues pretreated with SPI-0811 evaluated by immunofluorescence were protected from this effect, with localization of occludin to the apical epithelium similar to that of control tissues. Additionally, occludin remained in the detergent insoluble fraction in western analyses under the influence of SPI-0811 indicating localization to the TJs.

The next aim of the study was to investigate the role of Cl\textsuperscript{−} channels in gastro protection, given that SPI-0811 is a putative Cl\textsuperscript{−} secretagogue. Because ClC-2 has been shown to be localized to interepithelial tight junctions in intestinal studies\textsuperscript{[22]}, and in gastric mucosa in the parietal cells\textsuperscript{[29, 36]} is a target for the prostones, we were particularly interested in this channel. The CFTR served as an alternate possibility. Our data demonstrated that SPI-0811-mediated gastro protection against acid injury was attenuated by the ClC-2 inhibitor ZnCl\textsubscript{2} whereas the selective CFTR inhibitor 172 had no effect. Although CFTR is difficult to pharmacologically inhibit, our western analyses showed a lack of CFTR expression in porcine gastric mucosa as compared to robust expression in jejunal mucosa. In previous studies, investigators have shown that CFTR knockout mice are vulnerable to gastric ulcers due to loss of bicarbonate secretion. The lack of CFTR in porcine gastric mucosa especially in the fundus of stomach might partially explain susceptibility to acid injury in the present studies\textsuperscript{[32]}. Taken together; these studies suggest that activation of ClC-2 by SPI-0811 is integral to the mechanism of gastro protection. Further studies will be required to understand the mechanisms underlying these findings.
There are opposing views regarding the role of ClC-2 in gastric chloride secretion; some investigators have suggested involvement of ClC-2 in gastric acid secretion \cite{29, 36, 37} while another group has indicated that ClC-2 chloride secretion plays no role in production of HCl \cite{38}. It is also noteworthy that the apical ClC-2 channel has been suggested to serve as a route for both bicarbonate and Cl\textsuperscript{–} exit into the lumen \cite{29, 39}. This raises the possibility that ClC-2-mediated bicarbonate secretion attenuates acid injury to some extent by raising the pH. However in our studies, constant monitoring and adjustment of pH to a level of 1.5 would nullify this possibility. We also found that SPI-0811 produces a gastro protective effect not only via increasing gastric pH, but also by protecting and restoring the gastric mucus level (fig 2B). The importance of gastro protection via mucus protection has been previously shown \cite{7, 40, 34}.

ClC-2 localizes to the TJ in the intestine \cite{21, 27, 28, 41} and this localization facilitates the interactions with tight junction proteins and associated regulatory molecules. For example, recent studies have shown that ClC-2 is required for rapid reassembly of tight junction proteins after ischemic injury in murine intestine \cite{27}. The rapid process of repair is a hallmark of gastric mucosal barrier function, and may involve rapid restoration of tight junctions during restitution. Although this study does not completely answer questions as to the mechanism of SPI-0811, it does provide convincing evidence for the ability of SPI-0811 to protect the gastric mucosa against acid, and it does suggest this activity is at least in part attributable to a mechanism related to ClC-2 regulation.
**Figure 1:** Barrier function of acid injured porcine gastric mucosa to pretreatment of SPI-0811 (1μM). (A) Porcine gastric mucosa mounted on Ussing chambers challenged with mucosal acid (HCl, pH 1.5) over a 90 minute period had significantly lower TER when compared with control tissues bathed in normal Ringer’s (NR) (TER Ω.cm²=100 for acid-treated tissues versus 50 for control tissues). Mucosal application of the CIC-2 agonist SPI-0811 (1μM) prior to application of acid blocked reductions in TER (TER Ω.cm²=50 vs. 100). Values represent means ± SE, n=8, *p*< 0.01 vs. all other treatment groups. (B) As an alternative assessment of gastric mucosal barrier function, mucosal-to-serosal fluxes of ³H-mannitol were examined. In agreement with TER responses, ³H-mannitol flux was significantly elevated by treatment with acid (³H-mannitol flux 0.02 vs. 0.10 μM.cm²), and this response was inhibited by SPI-0811. (³H-mannitol flux 0.10 vs. 0.04 μM.cm²). Values represent mean ± SE, n=6, * p< 0.01.
Figure 2. Histological findings in acid-injured gastric mucosal tissues. (A) Tissues bathed in normal Ringer’s appeared uninjured after 90 minutes in Ussing chambers, whereas those exposed to acid had substantial evidence of epithelial injury. Pretreatment with SPI-0811 ameliorated injury evident in acid-injured tissues. Bar = 100µm. Each panel is representative of at least 3 separate animals. (B). PAS-Alcian blue staining findings in Control tissues (Figure 2.continued on page 70) bathed in Ringer’s solution for 90 minutes appeared to have normal expression of surface mucus by PAS –Alcian Blue staining whereas tissues exposed to acid had distinct loss to surface mucus staining. However, pretreatment with SPI-0811 prevented the loss of mucus in response to acid and thus preserved the surface mucus. (C). Immunofluorescence findings for tight junction protein occludin reveals Control tissues bathed in Ringer’s solution for 90 minutes appeared to have normal immunolocalization of the tight junction protein occludin (green) whereas tissues exposed to acid had very little visible apical epithelial occludin. However, pretreatment with SPI-0811 prevented the disorganized appearance of occludin in response to acid (Fig Continued from page 69).
Figure 3. Expression of TJ proteins in membrane fractions in acid injured porcine gastric mucosa. (A) Western analyses revealed expression of occludin in the detergent insoluble (DIS) in tissues treated with Ringer’s alone (NR), with very little discernable occludin in the detergent soluble fraction. On the other hand, treatment with acid (pH of 1.5 for 90 minutes) markedly reduced the expression of occludin in the detergent insoluble fraction, with a similar lack of occludin expression in the detergent soluble fraction. Pretreatment of tissues with mucosal SPI-0811 resulted in expression of occludin in the different fractions to a very similar extent as control tissues. (B) Western analysis revealed expression of whole occludin in either control, acid injured and SPI-0811 pretreated and acid injured porcine gastric mucosa from Ussing chambers. Acid injury markedly reduced expression of whole occludin compared to control while pretreatment with SPI-0811 preserved the loss of occludin from acid injury.
Figure 4. Electrical responses and $^3$H-mannitol fluxes of acid-injured porcine gastric mucosa. (A) Porcine gastric mucosa exposed to acid (pH 1.5 for 90 minutes) exhibited a significant drop in TER, which was completely blocked by pretreatment with SPI-0811. In attempt to discern which chloride channel was involved in the gastro protective mechanism of SPI-0811, ClC-2 and CFTR were inhibited. Addition of the ClC-2 inhibitor ZnCl$_2$ ameliorated the protective effect of SPI-0811, (TER $\Omega$.cm$^2$=100 vs.80) whereas the CFTR inhibitor CFTR inhibitor 172 had no effect. (TER $\Omega$.cm$^2$=100 vs. 100) Values represent mean ± SE, n=6; *$p<0.01$ vs. all treatment groups, #$p<0.05$ vs. all treatment groups. (B) As an alternate measure of barrier permeability mucosal-to-serosal of $^3$H-mannitol flux were performed, and reveled a significant increase in mannitol permeability in acid injured tissues (*$p<0.01$). Pretreatment with SPI-0811 blocked the increase in $^3$H-mannitol flux caused by acid injury, whereas blockade of ClC-2 inhibitor ZnCl$_2$ block the protective effect of SPI-0811($^3$H-mannitol flux $\mu$M.cm$^2$, 0.04 vs. 0.12, $p<0.05$) on permeability but CFTR inhibitor had no effect on the level of protective properties of SPI-0811 on permeability ($^3$H-mannitol flux $\mu$M.cm2, 0.04 vs. 0.05, $p<0.01$). Values represent mean±SE, n=6.
Figure 5: Expression of ClC-2 protein in porcine mucosa. Mucosal homogenates from porcine stomach were studied for expression of ClC-2 by western blotting. Expression of ClC-2 protein at its expected molecular weight (98KDa) was evident in all lanes. However, there appears to be greater expression of tissues bathed in Ringer’s solution as compared to those subjected to acid or acid and SPI-0811 (n=3). When the blocking peptide was used with the ClC-2 antibody, no bands were detected (upper panel).
REFERENCES


23. **Blikslager, AT, Roberts, MC, Rhoads, JM, Argenzio, RA.** Prostaglandins I2 and E2 have a synergistic role in rescuing epithelial barrier function in porcine ileum. *J Clin Invest* 1997; 100: 1928-1933 [PMID: 9329955 DOI: 10.1172/JCI119723].


27. **Nighot, PK, Blikslager, AT.** ClC-2 regulates mucosal barrier function associated with structural changes to the villus and epithelial tight junction. *Am J Physiol Gastrointest Liver Physiol* 2010; 299: G449-56 [PMID: 20489043 DOI: 10.1152/ajpgi.00520.2009].

28. **Nighot, PK, Moeser, AJ, Ryan, KA, Ghashghaei, T, Blikslager, AT.** ClC-2 is required for rapid restoration of epithelial tight junctions in ischemic-injured murine
DOI:10.1016/j.yexcr.2008.10.001].


CHAPTER IV

THE PROTECTIVE EFFECT OF CIC-2 AGONIST SPI-0811 ON
INDOMETHACIN–INDUCED EPITHELIAL BARRIER DYSFUNCTION IN
HUMAN GASTRIC EPITHELIAL CELLS
Abstract

Non-steroidal anti-inflammatory drugs like indomethacin are known to compromise gastrointestinal epithelial barrier. The mechanisms by which indomethacin affects the function of gastric mucosa, including epithelial tight junctions, are largely unknown. In prior studies, we have shown that prostones have a reparative effect on injured intestine, but we have not studied their potential gastro protective effect. We hypothesized that the chloride channel ClC-2 agonist, Cobiprostone (SPI-0811) would protect the gastric epithelial barrier exposed to indomethacin. Different doses of indomethacin (0.1, 0.25, 0.5, 0.75, and 1mM) were evaluated for their effect on barrier function as well as cytotoxicity in MKN28 gastric epithelial monolayer. Treatment with indomethacin (0.5mM) significantly decreased transepithelial electrical resistance (47% reduction in indomethacin-treated cells as compared to untreated control cells, \( p<0.05 \)). Paracellular permeability to FITC-labeled dextran was also significantly increased after indomethacin treatment (90% increases in permeability in indomethacin-treated cells versus control cells). Pretreatment of the MKN28 gastric cell line with cobiprostone significantly attenuated the decrease in TER caused by indomethacin to almost TER in control cells \( (p<0.05) \). The increase in the FITC-dextran permeability by indomethacin was reduced to 60% by pretreatment of Cobiprostone \( (p<0.05) \). Confocal microscopy revealed that indomethacin treatment markedly disrupted the immunolocalization of tight junction proteins occludin but not ZO-1 and other tight junction proteins, whereas pretreatment with Cobiprostone prevented indomethacin-induced disruption of occludin localization at the tight junction. The apoptosis caused by indomethacin was also prevented by Cobiprostone. In \textit{ex vivo} experiments, indomethacin caused significant drop in the TER in
the gastric mucosa from the wild type and CIC-2 knockout mice. Pretreatment with Cobiprostone attenuated indomethacin-induced loss of TER and increased paracellular permeability completely in wild type but not CIC-2 knockout mice. This data suggests that indomethacin compromises the gastric epithelial barrier via disruption of occludin, and this can be prevented by a CIC-2 agonist Cobiprostone.

Introduction

Gastropathy associated with the use of nonsteroidal anti-inflammatory drugs (NSAIDs) is one of the most frequent types of gastric mucosal injury (7, 8). To establish a clinical protocol for overcoming this side effect, the molecular mechanism governing NSAID-induced gastric mucosal injury needs to be elucidated. Inhibition of the synthesis of Prostaglandins (PGs), which have cytoprotective effects on gastric mucosa, has been thought to be the major mechanism of the gastro toxic effects of NSAIDs. However, evidence has suggested that the gastro toxic effects of NSAIDs cannot be explained only by their inhibitory effects on cyclooxygenase (COX) activity and PG synthesis. In order to reveal the nature of COX-independent mechanisms of NSAID-induced mucosal injury, a previous study has investigated gene expression up regulated by indomethacin (an NSAID) in a COX-independent manner in human gastric carcinoma (AGS) cells. This study has revealed that expression of genes encoding tight junction (TJ) proteins (claudin-4, claudin-1 and occludin) is up regulated by indomethacin (17). NSAIDS directly injure the gastric epithelial cells and thus alter tight junctions and can alter localization of the component proteins that constitute junctional complexes. In addition, NSAID have also been shown to compromise the
intercellular spaces directly beneath the tight junctions leading to further cell death and gastric bleeding. It is becoming evident that many patients suffer from chronic gastric ulcers, and these ulcers are increasingly being found in intensive care adult patients (8). This suggests that medications to provide gastro protection would reduce the morbidity of ulceration. Gastric ulcer disease either because of use of NSAIDS or some other factors associated with increase in acid secretion is a complex syndrome that involves epithelial barrier dysfunction, inflammation, cell proliferation, formation of granulation tissue, angiogenesis, apoptosis, and gastric bleeding. For decades, agents that suppress acid secretion (proton–pump inhibitors and H₂ receptor antagonists, HSP70 etc.) have been widely used for treatment of gastric ulcers. Gastro protection has also been studied in terms of effect of increased secretion of prostaglandin and ulcer healing (1, 3, 7, 10, 12, 25, 29-31). However, our understanding of gastro protection, which typically involves the simultaneous study of injury, remains incomplete. Furthermore, there is the prospect of novel pharmacological agents that might protect the stomach from acid induced and NSAID induced injuries, which could provide more options than proton pump inhibitors and antacids.

Gastric mucosal barrier function is essential for preventing potentially harmful elements present in the gastric lumen from gaining access to the gastric mucosa. The mucosal barrier is maintained by the TJs that are the most apical intercellular structures in epithelial cells and create a physiological barrier separating the apical and basolateral spaces. TJs contain transmembrane proteins such as occludin and the claudins, whose C-terminal regions interact with cytosolic proteins, such as zonula occludens-1 (16). Aside from injuring
epithelium NSAID directly causes disruption of the intraepithelial tight junction (TJ) protein complexes thereby increasing epithelial permeability, leading to apoptosis and thus ultimately cell death. The tight junction protein such as occludin has also been recently suggested to play a role as a signaling molecule in apoptotic pathway, beside its traditionally understood function as a structural protein.

NSAID and Acid injury remains an important component of gastric ulcer disease, despite more recent findings on the role of *Helicobacter pylori* that also contributes to this difficult problem. Models assessing the mechanisms of intestinal injury specifically with the use of NSAIDs have demonstrated that the critical event defining disruption of barrier function is the loss of TJ architecture and redistribution of TJ proteins such as occludin from the apical region of the interepithelial space (18). In our previous work, we have demonstrated a critical role for Cl⁻ secretion in the repair of intestinal barrier function in ischemia-injured porcine ileum. Furthermore, the barrier recovery was dependent on reassembly of apical tight junction complex, and was attributable to the activation of the Cl⁻ channel CIC-2, which is localized to the TJ (19). Interestingly, prior studies have shown that the nonselective secretory agonist PGE₂ triggered rapid recovery of transepithelial electrical resistance (TER) and reduced mucosal-to-serosal fluxes of ³H-mannitol in ischemic-injured, suggesting an important role of CIC-2 in intestinal barrier function (18). Given the previous findings that CIC-2 agonist lubiprostone enhances recovery of barrier function in ischemic-injured porcine intestine (19), and also role of CIC-2 in the development and maintenance of barrier function in the intestine (20, 21), we hypothesized that CIC-2 would play a critical role in the gastric mucosal barrier function. The present study was performed to evaluate the
ability of the novel ClC-2 agonist Cobiprostone to provide gastro protection using NSAID-induced injury in the human-adenocarcinoma gastric cell line model and ClC-2 knockout murine \textit{ex-vivo} model.

**Methods**

**Cell lines and culture conditions**

MKN28, a human gastric adenocarcinoma cell line was a generous gift from Dr. Richard Peek, School of Medicine, University of Tennessee. The cell line was propagated and maintained in RPMI-1640 media (Mediatech Inc.) that was supplemented with 10% FBS, 2.5 mg/ml amphotericin B, and 20 µg/ml gentamycin. The culture medium was changed every 48–72 hrs.

**Transepithelial electrical resistance**

MKN28 cells were plated on 12-mm diameter, 0.4-µm pore size tissue culture inserts (Corning) at 75% confluency. The resistance across the monolayer was measured using Epithelial Volt Ohm Meter (WPI, Sarasota, FL). The value obtained from a blank insert was subtracted to get the net resistance. The values of resistance were divided by the membrane area to get the resistance per unit area. The change in the electrical resistance whenever represented as the percent of baseline resistance was calculated as follows: percent baseline resistance = \[
\frac{[(\text{resistance from each time point}) - (\text{resistance from a blank insert})]}{[(\text{baseline resistance}) - (\text{resistance from a blank insert})]} \times 100,
\]

where baseline resistance was the resistance at the 0-min time point (24). When resistance was stable (at > 240 Ω·cm²), the culture medium from the upper (apical) compartment of the monolayer was removed and replaced with medium containing different doses of indoemthacin (100µM to 1mM).
control medium. After the dose response curve study, 500µM dose of indomethacin was used for all further experiments. In some experiments, monolayers were pretreated with either SPI-00811 (1µM for 60 min) or ZnCl₂ (300µM for 30 min) before the treatment with indomethacin. Cell viability was assessed by measuring the release of lactate dehydrogenase (LDH; LDH assay, Sigma) into the media. All data represent the average of more than three identically treated monolayers, and three individual experiments.

**Epithelial solute permeability**

The paracellular permeability in response to indomethacin (500µM) was also measured using FITC-labeled dextran (FITC-dextran, molecular weight: 4,000) as a permeable tracer that passes across the epithelial monolayer using the method described in the literature (23, 24). MKN28 cells were grown on the surface of 12mm-pore size tissue culture inserts. These cell monolayers were washed twice with HBSS and then placed on 24-well plates with 500 ml HBSS in the lower chamber. A 500µl aliquot of HBSS containing 5 mg/ml FITC-dextran was added to the luminal (apical) chamber, and the plates were placed in a CO₂ incubator at 37°C. After incubation for 30 min, a 100 µl samples was taken from the lower (basal) chamber, and the absorbance of FITC-dextran was determined at 494 nm using a spectrophotometer. Data were expressed as follows: FITC-dextran permeability index (%) = 

\[
\frac{(\text{experimental clearance}) - (\text{spontaneous clearance})}{(\text{clearance of filter alone}) - (\text{spontaneous clearance})} * 100.
\]

Permeability measurements were made under control conditions after an exposure to indomethacin (500µM), or after pretreatment with SPI-0811 (1µM for 60 min). All data represent the average of four identically treated monolayers.
Chemicals and Antibodies

Cobiprostone (SPI-8111, as frozen aliquots of 100 µM solutions in 100% DMSO) was obtained from Sucampo Pharmaceuticals, MD, USA. The primary antibodies used were Rabbit anti occludin, Mouse anti-ZO-1, Rabbit anti-claudin-2, Rabbit anti-claudin-3 (Invitrogen, Carlsbad, CA), Rabbit activated anti caspases-3 (Cell Signaling), and Rabbit anti ClC-2 (Alpha diagnostics, San Antino, Tx). Alexa 488-conjugated anti-mouse and Cy3-conjugated anti-rabbit IgG secondary antibodies and nuclear stain TO-PRO3 were purchased from Invitrogen.

GEL Electrophoresis and Western Blot Analysis

Gel electrophoresis and Western blot analysis was performed as previously described (20). Cell lysates were prepared by adding cell lysate buffer (50 mM Tris, 5 mM MgCl2·H2O, 25 mM KCl, 2 mM EDTA, 40 mM sodium fluoride, 4 mM sodium orthovanadate, 1% Triton X-100, and protease inhibitor cocktail (Roche)) directly to culture dish. The cells were scrapped, sonicated and centrifuged (10,000 RPM for 10 minutes) and the supernatant were stored at -80°C till further use. All the samples from cell lysates and tissue extracts were subjected to protein analysis using BCA Protein Assay Kit (Pierce, Rockford, IL). Tissue or cell extracts (amounts equalized by protein concentration) were mixed with appropriate volume of 2 × SDS-PAGE sample buffer and boiled for 4 min. Lysates were loaded on a 4-12% SDS polyacrylamide gradient gel, and electrophoresis was carried out according to standard protocols. Proteins were transferred to a PVDF membrane (Immobilon, Millipore, Billerica, MA) by using an electroblotting minitransfer apparatus (Biorad). Membranes were blocked at room temperature for 2-hours in 5% dry powdered milk in Tris-buffered saline.
plus 0.05% Tween 20 (TBST), and then incubated overnight in a primary antibody solution at 4°C. After washings in TBST, membranes were incubated with horseradish peroxidase conjugated secondary antibody, and the blots were developed for visualization of protein bands with luminol enhancer solution (Pierce, Rockford, IL).

**Immunofluorescence for tight junction proteins**

MKN28 cells were seeded onto 1.2-cm-diameter glass coverslips and allowed to get confluent. Following exposure to different experimental treatments, the monolayers were washed in cold PBS. The coverslips were fixed with methanol (30 min) and stored at -20°C until further staining. Cover slips were stained for occludin, ZO-1, and activated Caspases-3 using appropriate dilutions of the primary and secondary antibodies and the nuclear stain. The coverslips were mounted in fluorescent mounting medium and examined with a Nikon Eclipse 2000E inverted microscope equipped with the Nikon C1 confocal laser scanning system.

**Experimental Animals**

Studies were approved by the North Carolina State University Institutional Animal Care and Use Committee. Breeding pairs of heterozygous mice (ClC-2+/−) were a kind gift of Dr. James E. Melvin (University of Rochester, Rochester, NY). ClC-2-null (ClC-2−/−) and WT (ClC-2+/+) mice of 3 months of age were used in this study. All mice were maintained on a standard laboratory diet and the genotype of progeny mice was identified by PCR using primers specific for amplification of either intact or disrupted murine ClC-2 alleles (22).

**Ussing chamber studies**
The mice were euthanized by approved method, the entire stomach was clamped proximally and distally with Doyen intestinal forceps and placed in 10μM of indomethacin in oxygenated (95% O₂, 5% CO₂) ringer solution ((mM: 154 Na⁺, 6.3 K⁺, 137 Cl⁻, 0.3 H₂PO₄, 1.2 Ca²⁺, 0.7 Mg²⁺, 24 HCO₃⁻, pH 7.4) to block secretion of prostaglandins. The stomach was sharply incised at the lesser curvature and washed in ringer solution, and placed on 0.14-cm²- aperture Ussing chambers (18). The tissues were bathed on the serosal and mucosal sides with ringer solution. The serosal bathing solution contained indomethacin (5μM) and 10 mM glucose, which was osmotically balanced on the mucosal side with 10 mM mannitol. Bathing solutions were oxygenated (95% O₂-5% CO₂) and circulated in water-jacketed reservoirs maintained at 37°C. The spontaneous potential difference (PD) was measured with Ringer-agar bridges connected to calomel electrodes, and the PD was short circuited through AgCl electrodes with a voltage clamp that corrected for fluid resistance. Transepithelial electrical resistance (TER, Ω·cm²) was calculated from the spontaneous PD and short-circuit current. Indomethacin at the dose of 5μMwas added on the mucosal side with or without ClC-2 agonist SPI-0811 (1μM). After the equilibration period of 15minutes the experiments were run for up to 120 minutes. In each Ussing chamber experiment, duplicate tissues were studied from each animal and more than three animals were studied in each experimental group.

**Paracellular fluxes of [³H]-mannitol**

To evaluate mucosal permeability, 0.2 μCi/ml of [³H]-mannitol was added to the mucosal side of tissues mounted on Ussing chambers. Following a 15-min equilibration period, standards were collected from the mucosal side of each chamber and a 30-min mucosal-to-
serosal flux period was established by taking 0.5-ml samples from the serosal compartment. The presence of $^3$H was established by measuring $\beta$ emission in a liquid scintillation counter (2900 TR Liquid Scintillation Analyzer, PerkinElmer). Unidirectional $[^3]$H-mannitol fluxes from mucosal to serosal were determined based on the mannitol-specific activity added to the mucosal bathing solution and the net appearance of tritium over time in the serosal bathing solution, on a chamber unit area basis.

Results

Effect of Indomethacin on the transepithelial electrical resistance

MKN28 cells are human gastric epithelial cells that form confluent monolayers. To determine whether this was an appropriate model to investigate barrier function, we first established that MKN28 cell monolayers develop an increasing transepithelial resistance, a direct measurement of paracellular permeability that is regulated by intercellular tight junctions. MKN28 cells were grown to confluence and transepithelial electrical resistance (TER) was determined at different time intervals. We found that as MKN28 cells reached confluence, there was a progressive increase in TER, indicating that these cells form functional tight junctions. Next, we evaluated effect of indomethacin on the TER using different doses. Indomethacin at a dose of 100$\mu$M, 250$\mu$M, or 350$\mu$M did not significantly drop the TER after more than 12 hours of exposure. However, treatment of gastric epithelial MKN28 cell monolayer with higher doses of indomethacin (500$\mu$M, 700$\mu$M, and 1mM) caused a significant decrease in the TER at 4, 12, and 24 hours post treatment (Fig. 1). Indomethacin treatment at the dose rate of 700$\mu$M and 1mM but not 500$\mu$M caused considerable cell death, as determined by the trypan blue method and Trypan blue method.
Therefore, 500µM dose of indomethacin was used for further studies in evaluating the role of SPI-0811 in indomethacin-induced barrier dysfunction model.

**Effect of SPI-0811 on Indomethacin-induced permeability**

In the next set of experiments MKN28 cells were treated with SPI-0811 (1µM) or not and exposed to indomethacin (500µM). Pretreatment with the SPI-0811 significantly inhibited indomethacin-induced decrease in the TER (Fig. 2A). The paracellular permeability of MKN28 cells was also measured using FITC-dextran (molecular mass: 4,000). Indomethacin (500µM for 24 h) significantly increased the FITC-dextran permeability (Fig. 2B) of MKN28 cells, suggesting that indomethacin compromised paracellular barrier in these cells. Pretreatment with SPI-0811 (1µM) significantly attenuated indomethacin-induced increase in the FITC-dextran permeability. Thus the FITC-dextran permeability induced by indomethacin and the effect of SPI-0811 on indomethacin-induced permeability was found to be consistent with the data obtained from the TER measurements.

**SPI-0811 prevents indomethacin-induced loss of tight junction protein occludin**

To investigate the basis of reduction in the TER caused by indomethacin, the quantities of select TJ proteins occludin, ZO-1, claudin-1, and claudin-2, and apoptotic protein activated caspase-3 were determined by western blot analysis. There were no changes in the quantities of claudin-1, claudin-2 (data not shown), and ZO-1 (Fig. 3B) in the whole cell extracts from control or indomethacin treated MKN28 cells. On the other hand, indomethacin induced significant decrease in the quantity of occludin in whole cell lysates (Fig. 3A). Furthermore,
indomethacin-induced reduction in the quantity of occludin was attenuated when the cells were pretreated with SPI-0811. The quantities of claudin-1, claudin-2, and ZO-1 were not affected by SPI-0811 pretreatment alone. Additionally, indomethacin also induced significant increase in the expression of activated caspases -3 in the MKN28 cells and pretreatment with SPI-0811 inhibited this increase in the expression of activated caspases-3 (Fig. 5B).

In immunolocalization studies, occludin and ZO-1 were stained at apical tight junctions in control MKN28 cells, as determined by confocal immunofluorescence staining (Fig. 4). In indomethacin treated cells, however, localization of occludin at the junction was found to be disrupted. The loss of occludin from the TJ in the cells exposed to indomethacin was protected by pretreatment with SPI-0811. The ZO-1 immunofluorescence staining was not affected by indomethacin exposure alone or by the pretreatment with SPI-0811. In fluorescence studies, indomethacin-induced increase in the expression of activated caspases -3 was detected in MKN28 cells and pretreatment with SPI-0811 inhibited this increase in the expression of activated caspases-3 (Fig. 5A).

**Expression of ClC-2 in MKN-28 cells**

The literature is not very clear about expression of ClC-2 in the gastric mucosa. Thus western blot analysis was performed to study the expression of ClC-2 in MKN-28 gastric cells. We detected ClC-2 protein bands at around 98 kDa in the MKN-28 cells (Fig. 6). The specificity of this antibody has been established in the laboratory using specific binding antigen (data not shown).

**SPI-0811 prevents indomethacin-induced mucosal permeability in the gastric mucosa**
To determine if ClC-2 protein, indeed, is involved in the SPI-0811 mediated prevention of indomethacin-induced gastric mucosal injury, we conducted *ex vivo* studies using ClC-2<sup>+/+</sup> and wild type ClC-2<sup>-/-</sup> murine gastric mucosa. The gastric mucosa harvested from either wild type or ClC-2<sup>-/-</sup> mice were mounted in Ussing chamber and exposed to indomethacin with or without SPI-0811 pretreatment. Indomethacin (500 µM) caused significant drop in the TER in both wild type and ClC-2<sup>-/-</sup> gastric mucosa. Pretreatment of SPI-0811 (1µM) significantly attenuated the indomethacin-induced drop in TER in the wild type ClC-2<sup>+/+</sup> but not in ClC-2<sup>-/-</sup> gastric mucosa (Fig. 7A). Furthermore indomethacin also induced increases in the paracellular flux of mannitol in the ClC-2<sup>+/+</sup> as well as ClC-2<sup>-/-</sup> gastric mucosa. Pretreatment of gastric mucosa with SPI-0811 attenuated indomethacin-induced increase in the mannitol flux, to a greater extent in the ClC-2<sup>+/+</sup> mucosa compared to ClC-2<sup>-/-</sup> mucosa (Fig. 7B).

**Discussion**

In recent years the understanding of pathophysiology and treatment of peptic ulcers disease has changed remarkably. It is now evident that frequent use of NSAID in arthritis and other pain disorders can lead to loss of gastric mucosal barrier function and peptic ulcer disease. Loss of barrier function leading peptic ulcer disease remains one of the important components in the management of *Helicobacter pylori* infection. It is understood that the gastric barrier function plays an important role in preventing the gastric mucosa from noxious agents and also from the back diffusion of acid. NSAIDs are known to compromise intestinal barrier function via modulating tight junctions; similarly gastric barrier function has been also shown to be affected by NSAIDs. To prevent the gastric injury acid
suppressive drugs such as H₂ receptor antagonist and proton pump inhibitors are used to reduce the acidity and further damage to gastric mucosa. Though these drugs are highly effective in reducing acid secretion, improvement of mucosal barrier by specific modulators would be greatly helpful in protecting the gastric mucosa from injurious or noxious agents and further bacterial complications. Our laboratory has previously demonstrated an important role of chloride channel ClC-2 in modulating intestinal tight junction barrier function (18, 20, 22). Also another putative ClC-2 agonist prostanoid, lubiprostone has been shown to enhance intestinal barrier recovery after mucosal ischemic injury (19). Based on these previous works, we conducted present studies to establish a gastric mucosal injury model and study role of chloride channel ClC-2 in the gastric mucosal barrier.

In this study, we used the human gastric adenocarcinoma MKN-28 cells as our in vitro model to study effect of chloride channel ClC-2 agonist SPI0811in NSAID-induced gastric mucosal barrier dysfunction. We showed that NSAID indomethacin induced barrier dysfunction in a dose dependent manner. The dose of 500µm of NSAID indomethacin for 24 hours caused optimum reduction in the barrier function without overt cytotoxicity, and was used to evaluate protective properties of chloride channel ClC-2 agonist SPI-0811 against NSAID-induced barrier dysfunction. We found that indomethacin induced loss of the TER and increase in the paracellular permeability of dextran in MKN28 cells; both of these phenomena were prevented by the pretreatment of cells with ClC-2 agonist SPI-0811. Furthermore, we demonstrated that indomethacin compromised tight junction barrier via loss of tight junction protein occludin and not claudin-1, claudin-2, or ZO-1. Along with disruption of barrier function, apoptosis was also found to be induced by NSAID
indomethacin in MKN-28 cells. CIC-2 agonist SPI-0811(1µM) prevented the indomethacin-induced loss of tight junction barrier by preventing loss of occludin as well as preventing activation of pro-apoptotic caspases-3. Further evidence of role of CIC-2 in the gastric mucosal barrier was demonstrated in ex vivo experiments where CIC-2 knockout gastric mucosa was not protected by SPI-0811 in response to loss of barrier function caused by Indomethacin. These data suggest that SPI-0811 protected disruption of tight junction protein occludin via CIC-2-dependent mechanism that is necessary for protection of mucosal barrier function against indomethacin-induced gastric injury.

Although localization of CIC-2 is documented only in Rabbit gastric mucosa (27), we have been earlier able to demonstrate presence of CIC-2 in porcine gastric mucosa (manuscript under submission). However, role of CIC-2 in the gastric mucosa is not yet clear. Though earlier studies have suggested that CIC-2 is an apical chloride secretory channel (14, 15, 27), a recent study has shown no role of CIC-2 in the gastric secretion (11). CIC-2 knockout mice have been shown to have no significant difference in gastric acidification compared to wild type mice (4). On the other hand several ion transporters including Na\(^+\)-K\(^+\)-ATPase, the sodium-glucose cotransporter SGLT-1, sodium/hydrogen exchangers (NHEs), and the chloride channels cystic fibrosis transmembrane conductance regulator (CFTR) have been shown to regulate tight junction barrier (26). In present studies, the protective role of CIC-2 agonist SPI-0811 against indomethacin injured epithelial barrier function was found to be mediated via preservation of TJ barrier and prevention of occludin disruption. These findings are consistent with the protective properties of CIC-2 agonist lubiprostone against
intestinal epithelial injury (19).  ClC-2 has been shown to regulate intestinal barrier function via intracellular trafficking of occludin (20).

Our current study also provided evidence that indomethacin induced apoptosis in MKN28 cell line and this apoptosis was prevented by pretreatment with ClC-2 agonist SPI-0811. Several prior studies have shown involvement of indomethacin-induced apoptosis in gastric lesions. The mechanisms by which indomethacin induces apoptosis includes suppression of apoptosis inhibitor protein such as surviving (5), induction of reactive oxygen species-mediated mitochondrial pathology (13), or via induction of endoplasmic reticulum stress response that can be inhibited by heat shock protein Hsp70 (28). The mechanism by which ClC-2 agonist SPI0811 prevented indomethacin-induced apoptosis is not clear. However the cell membrane expression and channel activity of CIC-2 in enhanced under stress conditions (6) and in association with stress proteins such as heat shock protein Hsp90 (9). On the other hand, occludin has been shown to be essential for transducing apoptotic signals (32) and delocalized extrajunctional occludin forms complex with apoptic machinery including death inducing signaling complex (DISC), caspases 8 and 3, the death receptor FAS and the adaptor molecule FADD (2). This association of occludin with apoptic proteins helps extrusion of apoptic cells without loss of transepithelial resistance. Thus ClC-2 agonist SPI0811 mediated prevention of occludin disruption and apoptosis in response to indomethacin needs further investigation.
Our current data supports the idea that disruption of barrier function is the most important step in preventing further damage to the gastric mucosa including apoptosis and cell death in the NSAID-induced gastropathy and chloride channel CIC-2 could be a potential target for minimizing NSAID-induced gastric injury.
Indomethacin (100µM – 1mM) decreased transepithelial resistance in dose dependent manner. Indomethacin dose of 500µM, 700µM, and 1mM reduced the TER significantly at 4-hours post-treatment. *, # \( P < 0.05 \).
Figure 2. Effect of Cobiprostone (SPI-0811) on indomethacin-induced decrease in TER and increase in epithelial permeability in MKN-28 cells

A. Indomethacin (500µM) reduced the TER significantly at 4-hours post treatment, while pretreatment with Cobiprostone prevented the drop in TER caused by indomethacin (*p < 0.005).

B. Indomethacin (500µM) caused a significant increase in the paracellular permeability of dextran (4KD) in MKN-28 cells (24-hours post exposure), while pretreatment with Cobiprostone attenuated the indomethacin induced increase in the paracellular permeability (*p < 0.05).
Figure 3. Western expression of TJ proteins

A. Indomethacin exposure for 24 hours markedly reduced expression of whole occludin compared to control while pretreatment with SPI-0811 preserved the loss of occludin caused by indomethacin. B. Western analysis for zonula occludin1 revealed no changes after indomethacin treatment alone or with the pretreatment of SPI-0811.
**Figure 4.** Immunolocalization of TJ proteins

*A.* Localization of occludin at the tight junctions was disrupted in indomethacin exposed cells (500µM) while 1µM of Cobiprostone prevented indomethacin induced disruption of occludin (white arrows indicate tight junction area). MKN28 cells after pretreatment with Cobiprostone for 1 hour were exposed to indomethacin for 24 hours. Cells were fixed and stained for occludin (green) and nucleus (blue), and examined by confocal fluorescence, 90x.

*B.* Localization of zonula occludin 1 was not affected after treatment of MKN28 cells with indomethacin. MKN28 cells after pretreatment with Cobiprostone for 1 hour were exposed to indomethacin for 24 hours. Cells were fixed and stained for zonula occludin (green) and nucleus (blue), and examined by confocal fluorescence, 90x.
**Figure 5.** Expression of activated caspase-3

*A.* Confocal immunofluorescence detected increased expression of activated caspase-3 in the indomethacin treated MKN28 cells. Pretreatment with Cobiprostone prevented indomethacin induced activation of caspase-3. MKN28 cells after pretreatment with Cobiprostone for 1 hour were exposed to indomethacin for 24 hours. Cells were fixed and stained for activated caspase-3 and nucleus (blue), and examined by confocal fluorescence, 90x. *B.* Western blot analysis revealed reduced expression of activated caspase-3 in Cobiprostone treated cells compared to indomethacin treated cells alone.
Figure 6. Expression of ClC-2 in MKN-28 cells

MKN-28 cell homogenates were studied for expression of ClC-2 by western blotting. Expression of ClC-2 protein at its expected molecular weight (98kD) was evident in MKN-28 cells. The positive control of murine heart tissues revealed dense bands compared to MKN28 cells.
Figure 7. Effect of Cobiprostone (SPI-0811) on indomethacin-induced decrease in the transepithelial resistance in stomach mucosa

A. The gastric mucosa from ClC-2+/+ wild type and ClC-2−/− mice were mounted on Ussing chambers, as described in methods. Indomethacin (500µM) reduced TER significantly in ClC-2+/+ mice as well as ClC-2−/− mice compared to untreated tissues. Pretreatment with Cobiprostone prevented the drop in TER caused by indomethacin in ClC-2+/+ mice but not in ClC-2−/− mice (*p < 0.005). Pretreatment of Cobiprostone alone did not affect the TER (data not shown).

B. In the Ussing chamber experiments, as in A, indomethacin caused a significant increase in paracellular permeability of mannitol in the gastric mucosa in ClC-2+/+ and ClC-2−/− mice compared to untreated tissues. Pretreatment of Cobiprostone attenuated the indomethacin induced increase in the mannitol permeability in ClC-2+/+ mice to greater extent than ClC-2−/− mice (*p < 0.05).
References


CHAPTER V

INDOMETHACIN INDUCES GASTRIC EPITHELIAL BARRIER DYSFUNCTION
AND APOPTOSIS VIA p38 AND JNK IN MKN-28 CELLS
Abstract

Tight Junctions (TJ) create a paracellular barrier that is compromised when nonsteroidal anti-inflammatory drugs (NSAIDs) injure the gastric epithelium, leading to increased permeability. However, the mechanism of NSAID-induced gastric injury is unclear. Here, we examined the effect of the NSAID indomethacin on gastric mucosal barrier function and tight junctions in MKN-28 cells. In dose response studies, 500µm indomethacin induced an approximately 50% decrease in transepithelial resistance (TER; 45.7 vs. 24.4 Ω·cm² for control and indomethacin-treated cells respectively, \(p<0.05\)), and increased dextran permeability by approximately 90% \( (p<0.05)\). Both the indomethacin-mediated drop in TER and increase in dextran permeability were completely prevented by the p38 inhibitor (SB-203580) and a selective JNK- inhibitor (SP-600125), but not the MEK/ERK inhibitor (PD-98059). We found that all MAPKs were phosphorylated following indomethacin-induced injury but there was only a significant increase in phosphorylation of p38 MAPK and JNK as determined by western analysis and densitometry \( (p<0.05)\). In further western analyses of TJ proteins, expression of occludin was reduced by indomethacin, whereas there was no change in expression of claudin-2, claudin-4 and ZO-1. The loss of occludin expression induced by indomethacin was prevented by inhibition of p38 MAPK but not JNK or ERK. Confocal microscopic immunofluorescence revealed disruption of occludin localization at the site of the tight junction in indomethacin-treated cells, and this was attenuated by p38 MAPK inhibition. Since indomethacin is known to cause gastropathy via apoptosis, we also studied activation of caspases. Indomethacin did indeed induce apoptosis, as determined by evidence of activated caspase-3 immunofluorescence on confocal microscopy. This process was
attenuated by inhibition of p38 MAPK. Collectively this data suggests that indomethacin induces gastric epithelial barrier dysfunction by changes in occludin expression and localization and induction of apoptosis that is principally regulated by p38 MAPK.

**Introduction**

Use of nonsteroidal anti-inflammatory Drugs (NSAIDs) is known to cause gastrointestinal injury (1, 3, 10). In particular, the possibility of increased risk of gastrointestinal injury with chronic administration of NSAIDs for treatment of pain is the main concern. Various factors, including reduction in prostaglandins, increases in acid secretion, reduction in mucus secretion, disturbances in mucosal pH, increased production of oxygen free radicals, and compromises in mucosal barrier function have been found to play a role in the development of NSAID-induced gastric injury (32, 34, 35). However, the effects of indomethacin on the gastric epithelial tight junction (TJ) barrier are not well studied. TJs are the most apical component of intercellular junctional complexes and are composed of occludin, members of the claudin family of proteins, and junctional adhesion molecule (30). In gastric barrier function, besides TJ, mucus and endogenous prostaglandins also play a role in preventing mucosal injury and back diffusion of acid into the tissues. In the gastric mucosa, NSAIDs are known to cause mucosal injury in a cyclooxygenase (COX) dependent and independent manner. The NSAID indomethacin is also known to induce apoptosis in the gastric mucosa (13, 31). Experimentally, *H.pylori*, an organism involved in gastric ulceration has been shown to compromise gastric barrier via disrupting tight junctions (36). However, the
relationship between gastric ulcer pathogenesis, apoptosis, and TJ barrier disruption is unclear.

Mitogen-activated protein Kinases (MAPK) respond to extracellular stimuli and play a critical role in regulating cellular processes including proliferation, differentiation, apoptosis, and gene expression. The role of MAPKs in the regulation of the TJ barrier has been studied in various cell lines and tissues (5, 14, 16, 26). The role of MAPK in the TJ barrier, as reported in previous studies, has been specific to the cell line, tissue, and experimental injury model used. Recently, it has been shown that the NSAID aspirin causes damage in the gastric barrier by activating p38 MAPK via tight junction protein claudin-7 (20). Other than this report, there are no studies on the role of MAPKs in the gastric epithelial TJ barrier.

The goal of present study was to study if MAPKs have any role in indomethacin-induced changes in the gastric barrier. The results of our study show that, in vitro, indomethacin induces gastric barrier dysfunction in MKN28 human gastric cell line. Indomethacin induces loss of the TJ protein occludin and additionally causes apoptosis. Inhibition of p38 MAPK prevented indomethacin-induced loss of barrier function, disruption of occludin, and apoptosis. Alternatively, inhibition of JNK reduced loss of barrier function and ameliorated apoptosis while having no apparent protective role on TJs, and ERK inhibition had no effect. In ex vivo studies, indomethacin-induced barrier dysfunction in the murine gastric mucosa was prevented by inhibition of p38 MAPK.
**Materials and Methods**

**Cell line**

MKN28, a human gastric adenocarcinoma cell line, used for current experiments was a kind gift from Dr. Peek, University of Tennessee. The cell line was cultured using RPMI-1640 media (Sigma, St. Louis, MO), supplemented with 10% FBS, 2.5 mg/ml, amphotericin B, and 20 µg/ml gentamicin. The culture medium was changed every 48-72 hours.

**Reagents**

The primary antibodies used were mouse anti-occludin, mouse anti-ZO-1, rabbit anti-claudin-2, rabbit anti-claudin-3, rabbit anti-caludin-4 (Invitrogen, Carlsbad, CA), and rabbit activated anti-caspases-3 (Cell Signalling). Alexa 488-conjugated anti-mouse and Cy3-conjugated anti-rabbit IgG secondary antibodies were purchased from Invitrogen. Anti-phospho-p38 MAPK, p42/44 MAPK, and JNK antibodies, MEK inhibitor U-0126, p38 MAPK inhibitor SB-203580 and JNK inhibitor (SP-600125) were obtained from Promega (Madison, WI).

**Transepithelial electrical resistance**

MKN28 cells were plated on 12mm diameter, 0.4µm pore size tissue culture inserts (Corning) at 75% confluency. The transepithelial resistance (TER) was determined by a pair of electrodes positioned on the apical and basal sides of the monolayers and attached to an Epithelial Volt Ohm Meter (WPI, Sarasota, FL). For all transepithelial electrical resistance (TER) measurements, the inserts were plated at an equal density; the readings were taken in triplicate per monolayer and averaged. Percent change in baseline resistance was calculated as described (19): percent baseline resistance = [((resistance from each time point) -
(resistance from a blank insert)] / [(baseline resistance) - (resistance from a blank insert)] x 100, where baseline resistance was the resistance at the 0-min time point. Upon reaching a reasonably stable resistance (at >240 Ω.cm²), the culture medium from the upper (apical) compartment of the monolayer was removed and replaced with medium containing different doses of indomethacin (100µM to 1mM) or control medium. After the dose response study, 500µM dose of indomethacin was used for all further experiments. In respective experiments, monolayers were pretreated with U-0126 (10µM for 60 min), SB-203580 (10µM for 30 min), or SP-600125 (10µM for 30 min) before the addition of indomethacin (500µM). The data is represented as the average of more than three identically treated monolayers, from three independent experiments.

**Epithelial permeability**

The epithelial paracellular permeability in response to indomethacin was alternatively determined by FITC-labeled dextran (FD-4, molecular mass: 4,000; Sigma) as a permeable probe (19). Briefly, confluent MKN28 cell monolayers on tissue culture inserts were washed twice with HBSS and a 500µl aliquot of HBSS containing 5mg/ml FITC dextran was added to the apical chamber. After incubation of cell monolayers at 37°C for 30 min, a 100µl sample was taken from the lower chamber, and the absorbance of FITC-dextran was determined at 488nm using a spectrophotometer. The data of permeability, expressed as FITC-dextran permeability index (%) was determined as: - [(experimental clearance) - (spontaneous clearance)] / [(clearance of filter alone) - (spontaneous clearance)]-100. The permeability was determined either under control conditions, or after an exposure to
indomethacin (500µM), and after pretreatment with U-0126 (10µM for 60 min), SB-203580 (10µM for 30 min), or SP-600125 (10µM for 30 min).

**Immunofluorescence**

For immunofluorescence of TJ proteins, confluent monolayers of MKN28 cells on 1.2cm diameter glass coverslips were exposed to different experimental treatments. Followed by wash in cold PBS, the coverslips were fixed with methanol (30 min) and stored at -20°C until further staining. Cover slips were stained for occludin, claudins, ZO-1, and activated Caspases-3 using appropriate dilutions of the primary and secondary antibodies and the nuclear stain. The coverslips were mounted in fluorescent mounting medium and examined with a Nikon Eclipse 2000E inverted microscope equipped with the Nikon C1 confocal laser scanning system.

**Gel Electrophoresis and Western Blot Analysis**

Gel electrophoresis and western blot analysis was performed as previously described (17). Cell lysates were prepared by adding cell lysate buffer (50mM Tris, 5mM MgCl2·H20, 25mM KCl, 2mM EDTA, 40mM sodium fluoride, 4mM sodium orthovandate, 1% Triton X-100, and protease inhibitor cocktail (Roche)) directly to culture dish. The cells were scrapped, sonicated and centrifuged (10,000 RPM for 10 min) and the supernatant were stored at -80°C. All the samples from cell lysates and tissue extracts were subjected to protein analysis using BCA Protein Assay Kit (Pierce, Rockford, IL). Tissue or cell extracts (amounts equalized by protein concentration) were mixed with appropriate volumes of 2 × SDS-PAGE sample buffer and boiled for 4 min. Lysates were loaded on a 4-12% SDS
polyacrylamide gradient gel, and electrophoresis was carried out according to standard protocols. Proteins were transferred to a PVDF membrane (Immobilon, Millipore, Billerica, MA) by using an electroblotting minitransfer apparatus (Biorad). Membranes were blocked at room temperature for 2-hours in 5% dry powdered milk in Tris-buffered saline plus 0.05% Tween 20 (TBST), and then incubated overnight in a primary antibody solution at 4°C. After washings in TBST, membranes were incubated with horseradish peroxidase conjugated secondary antibody, and the blots were developed for visualization of protein bands with luminol enhancer solution (Pierce, Rockford, IL).

**Ussing chamber studies**

C57BL/6 wild type mice were obtained from a mouse colony maintained at NC State University. The mice were euthanized by an Institutional Animal Care and Use approved protocol, the entire stomach was clamped proximally and distally with Doyen intestinal forceps and placed in 10µM of indomethacin in oxygenated (95% O2/5% CO2) ringer solution ((mM: 154 Na+, 6.3 K+, 137 Cl-, 0.3 H2PO4, 1.2 Ca²+, 0.7 Mg²+, 24 HCO3⁻, pH 7.4) to block secretion of prostaglandins. The stomach was sharply incised at the lesser curvature and washed in ringer solution, and placed on 0.14-cm² aperture Ussing chambers ((18). The tissues were bathed on the serosal and mucosal sides with ringer solution. The serosal bathing solution contained indomethacin (5µM) and 10 mM glucose, which was osmotically balanced on the mucosal side with 10 mM mannitol. Bathing solutions were oxygenated (95% O2/5% CO2) and circulated in water-jacketed reservoirs maintained at 37°C. The spontaneous potential difference (PD) was measured with Ringer-agar bridges connected to
calomel electrodes, and the PD was short circuited through AgCl electrodes with a voltage clamp that corrected for fluid resistance. Transepithelial electrical resistance (TER, $\Omega \cdot \text{cm}^2$) was calculated from the spontaneous PD and short-circuit current. After the equilibration period of 15 minutes the experiments were run for up to 120 minutes. Indomethacin (500µM) was added on the mucosal side with or without pretreatment with MAPK inhibitors. In each Ussing chamber experiment, duplicate tissues were studied from each animal and more than three animals were studied in each experimental group.

**Statistical analysis**

All values are expressed as means ± SD. Data were analyzed using student’s t-tests or one-way ANOVA (significance, $p<0.05$).

**Results**

**Dose response for indomethacin-induced decreases in transepithelial electrical resistance (TER) and epithelial permeability of MKN-28 cells**

Indomethacin (100µM-1M) treatment of gastric epithelial MKN28 cell monolayers caused a dose-dependent decrease in the TER. Indomethacin at 100µM-300µM did not reveal any significant drop in the TER but indomethacin 500µM - 1000µM revealed a significant drop at 4, 12, and 24 hours after indomethacin exposure (Fig. 1). There was no evidence of any cytotoxic effects of indomethacin at the dose of 500µM at any time point but doses of 700µM - 1000µM caused significant toxicity, as determined by Trypan blue staining. Consistent with the TER data, indomethacin doses of 500µM -1000µM but not 100µM -300µM caused significant increases in the FITC dextran flux as soon as 4 hours post
exposure (data not shown). Based on the above findings, the 500µM dose of indomethacin was used in further studies.

**Effects of MAPK inhibitors on indomethacin-induced decreases in the TER and epithelial permeability in MKN-28 cells**

In the next set of experiments we studied the effects of MAPK inhibitors on indomethacin-induced decreases in TER. Indomethacin (500µM) reduced TER significantly at 4 hours post-treatment, while pre-treatment of cell monolayers with the p38 MAPK inhibitor SB-203580 (10µM) and the JNK inhibitor SP-600125 (10µM) prevented the drop in TER caused by indomethacin after 24 hours of exposure ($p < 0.05$) (Fig. 2A). However, the MEK inhibitor PD-98059 (10µM) did not have any effect on indomethacin-induced decreases in TER. Treatment of MKN28 cell monolayers with PD-98059 (10µM), SB-203508 (10µM), or SP-600125 (10µM) alone (for 24 hours) did not influence the baseline TER (data not shown). Similarly, 500µM indomethacin caused a significant increase in the epithelial permeability to FITC-dextran in MKN-28 cells (24 hours post exposure) while pretreatment of cell monolayers with the p38 MAPK inhibitor SB-203580 (10 µM) and JNK inhibitor SP-600125 (10µM) prevented the increase in permeability caused by indomethacin (Fig. 2B). Pretreatment with the MEK inhibitor PD-98059 (10µM) did not have any effect on indomethacin-induced increases in epithelial permeability. Furthermore, MAPK inhibitors PD-98059 (1-10µM), SB-203508 (1-10µM), or SP-600125 (10µM) alone (for 24 hours) did not have any effect on gastric epithelial permeability. Thus the TER and permeability data clearly indicated that p38 MAPK and JNK are involved in the indomethacin-induced gastric cell barrier dysfunction.
**Indomethacin reduces the expression of occludin and claudin-4**

To further deduce the effect of indomethacin on gastric cell barrier function, the expression of the TJ proteins occludin, claudin-2, claudin-3, claudin-4, and ZO-1 were determined by western blot analysis. There were no changes in the expression of ZO-1 and claudin-2 in total cell extracts after exposure to indomethacin (500 µM for 24 hours; Fig. 3). Claudin-3 was not detected in MKN-28 cell lysates. The expression of occludin and claudin-4 protein was found to be significantly reduced after exposure to indomethacin (Fig. 3). Furthermore, the indomethacin-induced decrease in the expression of occludin was attenuated when cell monolayers were pretreated with SB-203580. SB-203580 (10µM) pretreatment did not change the expression of claudin-4. Indomethacin-induced decreases in the expression of occludin and claudin-4 but were not changed by the pretreatment with PD-98059 and SP-600125.

**p38 MAPK and not JNK and ERK mediates indomethacin-induced disruption of occludin**

We found that exposure of MKN28 cell monolayers to indomethacin induced phosphorylation of ERK, p38 MAPK, and JNK. Indomethacin treatment resulted in marked phosphorylation of p38 MAPK compared to control untreated cells (Fig. 4). The indomethacin-induced phosphorylation of p38 MAPK was blocked by pretreatment with the p38 MAPK inhibitor, SB-203580. Similarly, indomethacin-induced phosphorylation of JNK was significantly increased compared to control and was blocked by SP-600125 (Fig. 4). ERK was found to be phosphorylated in untreated MKN28 cells, possibly because of the
presence of serum in the culture media. Indomethacin treated cells also showed phosphorylation of ERK which was not significantly different from the control cells.

In further experiments, we studied morphological evidence for the role of MAPK in indomethacin-induced barrier dysfunction by evaluating immunolocalization of the TJ protein occludin. Confocal immunofluorescence revealed that indomethacin treatment disrupted localization of occludin at the tight junctions (Fig. 5). Furthermore, the indomethacin-induced disruption of occludin was attenuated by inhibition of p38 MAPK but not JNK or ERK (Fig. 5). This data indicated that the epithelial barrier dysfunction caused by indomethacin was mediated at least in part by activation of p38 MAPK which in turn compromises localization of the tight junction protein occludin.

**Indomethacin induces apoptosis via p38 MAPK activation and disruption of occludin**

In further studies, we found that 500µm of indomethacin increased expression of caspase-3, as seen in the western blot (Fig. 6A). This increased expression of caspase-3 was inhibited by pretreatment with the p38 MAPK inhibitor. Using confocal immunofluorescence, the presence of caspase-3 was detected in indomethacin-treated cells. In cells pretreated with the p38 MAPK inhibitor caspase-3 immunofluorescence was significantly reduced compared to the cells treated with indomethacin alone (Fig. 6B). Thus, p38 MAPK activation by indomethacin appears to be a crucial step in indomethacin-induced apoptosis. Overall, indomethacin-induced disruption of occludin was prevented inhibition of p38 MAPK and indomethacin-induced activation of caspase-3 was also prevented by inhibition of p38 MAPK; however, it is not clear at this point if there is a link between these two processes.
The closer look at the occludin and caspase-3 co-immunofluorescence, however, indicated that activated caspase-3 is mostly localized to the region where disruption of occludin is quite obvious. In indomethacin treated cell, ERK inhibition did not prevent activation of caspase-3. Alternatively, JNK inhibition prevented activation of caspase-3, but disruption of occludin was still present (Fig. 6).

**Effect of MAPK inhibition on indomethacin-induced mucosal permeability in the gastric mucosa**

To determine if MAPK signaling is involved in indomethacin-induced gastric mucosal injury, we conducted *ex vivo* studies using murine gastric mucosa. The gastric mucosa was harvested from wild type mice and was immediately mounted in Ussing chamber and exposed to indomethacin with or without pretreatment of MAPK inhibitors. Indomethacin (500µM) caused a significant drop in the TER in murine gastric mucosa. Pretreatment with the P38 MAPK inhibitor and the JNK inhibitor significantly attenuated the indomethacin-induced drop in TER in the mucosa, but ERK inhibition had no effect (Fig. 7A). Furthermore indomethacin also induced increases in the paracellular flux of mannitol in the gastric mucosa and pretreatment with p38 MAPK inhibitor and JNK inhibitor attenuated indomethacin-induced increase in the mannitol flux significantly while ERK inhibition did not change the increased permeability caused by indomethacin (Fig. 7B).
Discussion

Nonsteriodal anti-inflammatory drugs (NSAID) are the most widely used therapeutic agents in the treatment of pain, inflammation, and fever (29). The anti-inflammatory action of NSAIDs is mediated through the inhibition of cyclooxygenase (COX). COX is an enzyme essential for the synthesis of prostaglandins, which have a strong propensity for inducing inflammation. Prostaglandins, such as prostaglandin E2 (PGE2) inhibit apoptosis and stimulate cell growth, angiogenesis, and metastasis (11). However, several lines of evidence suggest that gastropathy by NSAIDs also involves COX-independent mechanisms (23, 24). For instance, NSAIDs caused apoptosis and the inhibition of cell growth in COX null fibroblasts and tumor cells in which COX expression was absent (9, 38). Indomethacin, as well as other NSAIDs, induces gastric epithelial damage (13, 31). To understand the mechanism that is involved in indomethacin-induced gastric damage might provide a new strategy for the prevention of indomethacin-associated gastropathy, reduce side effects, and clinical morbidity. In the present study, we examined how paracellular junctional permeability changed when gastric epithelial cells and gastric mucosa were exposed to indomethacin in vitro and ex vivo, respectively. In our studies, we found that indomethacin at the dose of 500µm reduced the TER in MKN-28 gastric epithelial cells and increased solute permeability (Fig. 1 and 2). We initially performed dose response experiments to examine the effects of indomethacin on TER in the MKN-28 cell line, and observed indices of damage (changes in TER) as the dosage escalated above the 100µM range. Higher concentrations of indomethacin have been reported to be needed to induce gastropathy in prior studies (33). Other studies using oral administration of indomethacin and aspirin in rats have shown that
the concentrations in the stomach were 1–8 and 10–100 µM, respectively (7). We found that 500 µm dose of indomethacin induced significant decrease in TER increase in permeability at 4 hours post treatment without cytotoxic effects. The same dose of indomethacin induced significant drop in the TER and increased mucosal permeability in our ex vivo studies, and is within the in vivo physiological range.

In further experiments we demonstrated that p38 MAPK and JNK are involved in indomethacin-induced loss of barrier function in the gastric cell line, and this loss of barrier occurs through loss of the TJ protein occludin as well via apoptosis. The protective effect of inhibition of p38 MAPK on the mucosal barrier was further demonstrated in ex vivo studies using murine gastric mucosa. The role of intracellular signaling pathways in the regulation of TJ permeability has only recently begun to be elucidated. Studies published so far have focused largely on the MEK-ERK pathway. Specifically, it has been reported that growth factor-dependent activation of ERK1/2 causes an increase in TER associated with modulation of claudin expression in MDCK II cells (6, 15, 28) as well as in T84 intestinal epithelial cells (12). Although the involvement of p38 MAPK in the regulation of epithelial barrier function has been less thoroughly investigated, circumstantial evidence indicates that activation of this MAPK may increase TJ permeability. Thus, inhibition of p38 MAPK was reported to prevent the disruption of the TJ barrier induced by various stimuli in different epithelial cell types (20, 22). Similarly, inhibition of JNK signaling has been shown to reduce claudin-2 expression in MDCK II cells (4, 8). The role of MAPK in epithelial permeability has been emphasized in several studies. Activation of ERK1/2 induced H₂O₂-mediated permeability with rearrangement of TJ protein occludin in endothelial HUVEC cells (14).
Similarly, the role of JNK in osmotic stress induced barrier dysfunction in the intestinal epithelium (25) and the role of ERK MAPK in the H$_2$O$_2$-mediated intestinal epithelial cell barrier dysfunction has been demonstrated (27). In a gastric cell line MKN-28, Oshima et al., (20) have specifically shown that aspirin-induced epithelial barrier permeability via tight junction protein claudin-7. Aspirin phosphorylated MAPKs, including p42/44 MAPK, p38 MAPK, and JNK. However, only the p38 MAPK inhibitor SB-203580 attenuated the aspirin-induced decrease in TER and aspirin-induced increase in permeability. Although the role of MAPKs in epithelial barrier function and TJ function appears to be clear, the underlying mechanism and involvement of specific MAPK components varies, possibly due to the cell type or the stimulant involved in the experimental studies.

Our current study also provided evidence that indomethacin induced apoptosis in MKN28 cell line and this apoptosis was prevented by pretreatment with p38 MAPK inhibitor SB-203580 (Fig 8). Several prior studies have shown involvement of indomethacin-induced apoptosis in gastric lesions. Among several mechanisms by which indomethacin induce apoptosis is an induction of endoplasmic reticulum stress response (31). It has also been shown that activation of p38 MAPK is one of the important components in the activation of endoplasmic reticulum stress (21). In our studies, along with prevention of occludin disruption, inhibition of p38 MAPK also prevented indomethacin-induced activation of caspase-3, and the presence of activated caspase-3 was detected in the membrane areas where occludin has been disrupted. Recently it has been shown that normal apoptosis or caspase-3 activation required occludin in experimentally TNF-α-induced apoptosis. On the other hand, occludin has been shown to be essential for transducing apoptotic signals (37) and
delocalized extrajunctional occludin forms a complex with apoptotic machinery including death-inducing signaling complex (DISC), caspases-8 and -3, the death receptor FAS and the adaptor molecule FADD (2). Thus it is possible that p38 MAPK inhibition is preventing the downstream signaling for apoptosis where occludin may act as one of the signaling molecules inducing the apoptosis.

The present study shows that even though indomethacin phosphorylates p38 MAPK, JNK, and ERK, indomethacin-induced reductions in TER and increased permeability was only attenuated by inhibition of p38 MAPK and JNK. We also found that occludin expression and localization was preserved by inhibition of p38 MAPK and not JNK. Though it appears that JNK was involved in indomethacin-induced loss of TER, its specific action on TJ was not studied. It is quite possible that, as reported previously (4), JNK mediates TJ barrier function via specific claudin isoforms not examined in this study. Our current data support the idea that disruption of barrier function involves MAPK activation and a specific tight junction protein is targeted by particular signaling components of the MAPK pathways. Thus p38 MAPK could be a potential therapeutic target in preventing indomethacin induced damage to the gastric mucosa.
Figure 1. Dose curve for indomethacin-induced loss of TER in MKN-28 cell line.

Indomethacin (100µM – 1mM) decreased transepithelial resistance in dose dependent manner. Indomethacin dose of 500µM, 700µM, and 1mM reduced the TER significantly at 4-hours post-treatment. *p < 0.05.
Figure 2. Effect of MAPK inhibition on indomethacin-induced changes in the epithelial barrier in MKN-28 cells

A. Indomethacin (500µM) reduced the TER significantly at 4-hours post treatment, while inhibition of p38 and JNK MAPK significantly attenuated the drop in TER caused by indomethacin (*p < 0.005).

B. Indomethacin (500µM) caused a significant increase in the paracellular permeability of dextran (4KD) in MKN-28 cells (24-hours post exposure), while consistent with effect on TER, p38 and JNK MAPK inhibition attenuated the indomethacin induced increase in the paracellular permeability (#, different from Indo alone, p < 0.05).
**Figure 3.** Western analysis of Tight Junction proteins

Indomethacin exposure for 24 hours markedly reduced expression of occludin and claudin-4 but not claudin-2 and zona occludin1. Inhibition of p38 MAPK attenuated loss of expression of occludin caused by indomethacin.
**Figure 4.** Phosphorylation of MAPK

Indomethacin induced phosphorylation of ERK, p38 MAPK, and JNK in MKN28 cells. The indomethacin-induced phosphorylation of p38 MAPK and JNK MAPK was blocked by pretreatment with the p38 MAPK inhibitor, SB-203580 and JNK inhibitor SP-600125, respectively. Indomethacin treated cells, though, showed phosphorylation of ERK, it was not significantly different from the control cells.
Figure 5. Immunolocalization of TJ protein occludin

Immunolocalization of occludin at the tight junctions was found to be disrupted in indomethacin exposed cells (500µM) (arrow) while inhibition of p38 and not JNK and ERK MAPK prevented indomethacin induced disruption of occludin. MKN28 cells after pretreatment with MAPK inhibitors for 1 hour were exposed to indomethacin for 24 hours. Cells were fixed and stained for occludin (green) and examined by confocal fluorescence, 90x.
Figure 6. Expression of activated caspase-3

A. Western analysis revealed increased expression of activated caspase-3 in the indomethacin treated MKN28 cells. Pretreatment with p38 and JNK MAPK inhibitors prevented indomethacin induced expression of activation of caspase-3. B. In confocal immunofluorescence, the presence of caspase-3 was detected in indomethacin-treated cells. In cells pretreated with the p38 MAPK inhibitor, caspase-3 immunofluorescence was significantly reduced (upper row, 90x; middle row, 10x). Also, activated caspase-3 was mostly localized to the region where disruption of occludin was quite obvious (arrow, bottom row, 90x). Though, JNK inhibition also prevented activation of caspase-3 (panel A), disruption of occludin was still present (panel B, upper row). MKN28 cells after pretreatment with MAPK inhibitors for 1 hour were exposed to indomethacin for 24 hours. Cells were fixed and stained for activated caspase-3 (red) and occludin (green), and examined by confocal fluorescence.
Figure 7. Effect of MAPK inhibition on indomethacin-induced alterations in the gastric barrier function

A. The gastric mucosa from wild type mice were mounted on Ussing chambers, as described in methods. Indomethacin (500µM) reduced the TER significantly (*p < 0.05, compared to control cells) while pretreatment with p38 and JNK MAPK inhibitors prevented the drop in TER caused by indomethacin. B. In the Ussing chamber experiments, as in A, indomethacin caused a significant increase in paracellular permeability of mannitol in the gastric mucosa compared to untreated tissues (*p < 0.05). In the gastric mucosa pretreated with p38 and JNK MAPK inhibitors before the treatment of indomethacin, the mannitol permeability was comparable to that of control gastric mucosa.
References


5. Chen Y, Lu Q, Schneeberger EE and Goodenough DA. Restoration of tight junction structure and barrier function by down-regulation of the mitogen-activated protein kinase


