ABSTRACT

SHIFFLETT, DONNIE EDWARD. REGULATION OF COX-2 AND PGE\textsubscript{2}-DEPENDENT RECOVERY OF INTESTINAL BARRIER FUNCTION.
(Under the direction of Anthony Thomas Blikslager and Samuel L. Jones)

The regulation of intestinal barrier function is of importance in various clinical situations. For example, in cases of ischemia/reperfusion injury, there is a disruption in the gastrointestinal barrier. This may lead to release of pro-inflammatory cytokines and neutrophil (PMN) infiltration. It has been demonstrated that COX-2, which is upregulated during inflammation, and its downstream product PGE\textsubscript{2} are important for recovery of barrier function, yet their regulation has not been fully characterized. **Study 1:** To study the effects of PMNs on acutely injured mucosa, we applied PMNs isolated from circulation to ischemia-injured porcine ileal mucosa. Since COX-2 is upregulated by inflammatory mediators such as IL-1\textbeta, which is released by PMNs, we postulated that PMNs enhance recovery of ischemia-injured mucosa by a pathway involving IL-1\textbeta and COX-2. Application of 5x10\textsuperscript{6} PMNs to ischemia-injured ileal mucosa significantly enhanced transepithelial resistance (TER), an effect inhibited by the selective COX-2 inhibitor NS-398 (5\textmu M) and an IL-1\textbeta receptor antagonist (0.1mg/ml). Western blots revealed up-regulation of COX-2 in response to PMNs, which was inhibited by an IL-1\textbeta receptor antagonist. Real time PCR revealed increased mRNA COX-2 expression, which preceded increased COX-2 protein expression in response to IL-1\textbeta. We concluded that PMNs augment recovery of TER in ischemia-injured ileal mucosa via IL-1\textbeta-dependent upregulation of COX-2. **Study 2:** Mitogen activated protein kinase (MAPK) pathways transduce signals from a diverse array of extracellular stimuli, including IL-1\textbeta. The three primary MAPK signaling pathways are
the extracellular regulated kinases (ERK 1&2), p38 MAPK, and c-Jun NH₂-terminal kinase (JNK). Because COX-2 expression is regulated in part by MAPK’s, we postulated that MAPK pathways would play an integral role in recovery of porcine ischemia-injured ileal mucosa. Treatment of tissues with the p38 MAPK inhibitor SB-203580 (0.1mM), or the ERK 1&2 inhibitor, PD-98059 (0.1mM), abolished recovery. Western blots revealed that SB-203580 inhibited upregulation of COX-2 whereas PD-98059 had no effect on COX-2 expression. Inhibition of TER by SB-203580 or PD-98059 was overcome by exogenous PGE₂ (1µM). The JNK inhibitor, SP-600125 (0.1mM), significantly increased TER and resulted in COX-2 upregulation. Thus, COX-2 expression appears to be positively and negatively regulated by the p38 MAPK and the JNK pathways respectively. Alternatively, ERK 1&2 appears to be involved in COX-2-independent reparative events. **Study 3:** In previous studies, we had shown that PGE₂ restored barrier function via a signal transduction pathway involving Cl⁻ secretion and recovery of interepithelial tight junctions (TJs). To study these mechanisms, we utilized human colonic T84 cells. We postulated that PGE₂ induced chloride secretion would precede increases in TER associated with re-distribution of critical proteins to TJs. T84 cells were grown to confluence, but utilized at lower TER values (200-500 ohms.cm²) to simulate our ileal mucosal model of “leaky” restituted epithelium. Basolateral application of PGE₂ (1µM) induced transient increases in Isc, indicative of chloride secretion, followed by sustained increases in TER. Basolateral application of the Na⁺/K⁺/2Cl⁻ cotransporter inhibitor bumetanide (0.1mM), abolished the PGE₂-induced rise in Isc and subsequent elevations in TER. PGE₂ induced a shift in claudin-1 from the Triton-X soluble to insoluble fraction, beginning 4-hour after PGE₂ administration, which was prevented by bumetanide. Alternatively, there were no changes in occludin or claudins-3 and
-5. Immunoflourescence demonstrated that PGE$_2$ increased accumulation of claudin-1 at the apical lateral membrane. Additionally, we showed that PGE$_2$ increased tyrosine phosphorylation of claudin-1 within 30 min, an effect prevented by bumetanide. Therefore, PGE$_2$-induced chloride secretion in T84 cells is directly linked with increases in TER, and these elevations in TER are associated with phosphorylation of claudin-1 and a shift in claudin-1 to the tight junction.
REGULATION OF COX-2 AND PGE$_2$-DEPENDENT RECOVERY OF INTESTINAL BARRIER FUNCTION

By

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BIOGRAPHY

I grew up in Lewistown, Pennsylvania, a small rural town located in central PA, near Penn State University. I attended Lock Haven University, a Division II school in PA, where I received a B.S. in Health Science in May 1996. After graduation, I continued on with my education at Virginia Tech. In May 1998 I received my M.S. in Exercise Physiology from Virginia Tech. After receiving my M.S., I worked for a little over one year in the Washington D.C. area utilizing my degree. In August 1999, I went back to school for my Ph.D. at Virginia Tech. Due to the retirement of my advisor at Virginia Tech, I left Virginia in May 2001 and enrolled in the Ph.D. program at North Carolina State University under the direction of Anthony Blikslager.
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CHAPTER ONE

THE REGULATION OF THE TIGHT JUNCTION AND INTESTINAL BARRIER FUNCTION
Introduction

The tight junction (TJ) of epithelial cells is the most apical of the intercellular junctions and is highly regulated. There are several mechanisms by which TJ’s can be influenced and controlled. One physiological regulator of TJ permeability is small intestinal Na\(^+\)-nutrient cotransport. For example, initiation of Na\(^+\)-glucose transport in isolated small intestine induces increases in paracellular flux and dilation of the TJ. Cytokines, which serve as extracellular signals in many pathophysiological conditions, have also been shown to influence intestinal barrier function. The potential for understanding cytokines and gastrointestinal permeability could lead to novel treatment options in a variety of clinical diseases. For example, in cases of inflammatory bowel disease, bacterial infections, HIV, and surgery, there is a disruption in the gastrointestinal barrier. This may lead to activation of macrophages, neutrophil infiltration, release of pro-inflammatory and anti-inflammatory cytokines and ultimately disruption of the intestinal barrier. Selectively blocking some cytokines while possibly up regulating others could lead to an improved defense mechanism and/or healing. There is also a growing interest in understanding not only the signaling pathways responsible for loss of barrier function but also recovery of barrier function. Our laboratory is attempting to understand the mechanisms by which PGE\(_2\) is capable of enhancing recovery of tight junctional structure in damaged intestinal mucosa.

Tight Junction Structure

The tight junction (TJ) of epithelial cells forms a belt-like interconnected series of junctional strands of protein connecting epithelial cells. Tight junctions separate the apical from the basal and lateral plasma membranes (fence function) and are therefore responsible
for polarizing epithelial cells. Polarization of epithelial cells is necessary for movement of ions, water, and macromolecules between compartments as each membrane contains different ion channels, enzymes, and transport proteins. For example, Na⁺/K⁺/ATPase is localized to the basolateral surface to allow the cell to develop a net charge across the cell and this is the principal driving force for cellular transport processes. Tight junctions are also believed to be the rate-limiting barrier to paracellular transport (gate function).(24,26) Paracellular transport is a passive process and works in conjunction with the transcellular active transport processes. Thus, the paracellular route works in compliment with transcellular processes to determine the overall barrier properties and net transport of ions and solutes. It has also been demonstrated that TJ’s are dynamic structures regulating the transport of water and solutes.(3) Permeability across the TJ is regulated by numerous mechanisms, including cytoskeletal tone.(41,43) Occludin, claudins, and junction adhesion molecule (JAM) form the transmembrane component of the TJ, and result in the sealing of the paracellular space.(20,21,62) Thus far, there have been more than 20 members of the claudin family of proteins identified and it appears that the claudins are responsible for formation of TJ strands and creating the TJ’s physiological barrier.(20,22,31,73) Although occludin is also associated with the TJ, it has been shown that expression of occludin in MDCK cells does not alter the number of strands of the TJ as assessed by freeze fracture analysis.(5) Structurally, claudins and occludin have two extracellular loops. For claudins, the first loop is larger and more hydrophobic than the second loop. Additionally it has been demonstrated that the first extracellular loop of claudins-2 and –15 create a charge selective channel in the paracellular space.(15,16,22) Some of the claudins have restricted expression based upon cell type. For example, claudin-5 is expressed only in endothelial TJ’s,(52)
claudin-16 in the Loop of Henle of the kidney(67), and claudin-11 in the myelin sheaths of the central nervous system and sertoli cells in the testis.(51) Evidence for claudins regulating barrier function include studies in MDCK cells in which overexpression of claudin-1 increased TER and decreased paracellular flux of 4 and 40kDa FITC labeled dextrans.(33) Overexpression of epitope tagged and non-epitope tagged claudin-1 also results in enhanced transepithelial resistance (TER).(50) Evidence that claudins have charge selectivity has been demonstrated via work with claudin-16 (paracellin-1). As mentioned previously, claudin-16 has a restricted pattern of expression to the ascending loop of Henle. Claudin-16 is required for the paracellular reabsorption of Mg$^+$ in the kidney.(67) The importance of this protein is demonstrated in humans with a genetic defect encoding for the claudin-16 gene. Lack of claudin-16 expression leads to decreased Mg$^+$ and Ca$^+$ reabsorption. The ensuing increase in urinary Ca$^+$ predisposes to renal stone formation and often these patients require transplantation. Coinciding with the idea that claudins have a role in transport functions, claudins are expressed at various levels in different organs as well as having differential expression patterns within organs. For example, claudin-2 is expressed almost exclusively in the crypts of the small intestine, while claudin-4 is expressed almost exclusively in the villi of the small intestine. These differential expression patterns could be functionally important for transport functions.(60) As an example, overexpression of wild-type claudin-4, but not mutant claudin-4 in MDCK cells resulted in decreased permeability of Na$^+$ compared with Cl$^-$ ions.(15,74) Furthermore, mutation of claudin-15 created a Cl$^-$ selective pathway compared to Na$^+$. Conversely, wild-type claudin-15 exhibited the opposite charge selectivity.(16)
Transmembrane proteins are associated with an intracellular cluster of proteins referred to as the cytoplasmic plaque. These proteins are associated with assembly and scaffolding of the paracellular space. The primary structural proteins of this family of cytosolic plaque proteins are ZO-1(71), ZO-2(25,36), ZO-3(29), cingulin(14), and 7H6.(81) Furthermore, the ZO family of proteins are capable of binding with each other(18,29), the actin cytoskeleton(18) and junction adhesion molecule.(17) Furthermore, the carboxyl terminals of both occludin and claudins can bind ZO-1, -2, -3.(18,29,35,76) Thus, it has been speculated that the ZO’s are important for targeting claudins and occludin to the TJ.

Yet another structural component of the TJ is the actin cytoskeleton. Evidence suggesting that actin regulates the TJ has been shown by disruption of actin with cytochalasin. This results in a decrease in the size of TJ fibrils(40,56) and also results in declining epithelial barrier function. Furthermore, introduction of a constitutively activated myosin light chain kinase in MDCK cells results in contraction of the cytoskeleton, decreased TER, and increased paracellular flux.(30)

**Regulation of the Tight Junction**

The regulation of occludin has been recognized as being important for TJ regulation. Overexpression of occludin has been associated with increases in TER in MDCK cells.(63) Furthermore, phosphorylated forms of occludin have been shown to be associated with the TJ(4,13,19,63) Phosphorylation of this protein has been shown to occur on serine and only weakly on threonine.(72) However, a more recent study has shown that occludin is phosphorylated on threonine and tyrosine residues.(78) Furthermore, Yoo et al. have shown that TER can be improved upon exogenous administration of the nonphorbol PKC agonist,
bryostatin, and this improvement in TER is due to phosphorylation and mobilization of tight junctional proteins from a Triton X-100 soluble fraction to a Triton X-100 insoluble fraction. Triton X-100 solubility or insolubility is an operational definition, which has been utilized to describe proteins not associated with the cytoskeleton or associated with the cytoskeleton, respectively. (64) Phosphorylation of ZO-1, which occurs on tyrosine and serine residues, is also important for barrier function. (68) ZO-1 phosphorylation has been demonstrated to be enhanced in low TER MDCK cells when compared to high TER MDCK cells. (70) Additionally, ZO-1 tyrosine phosphorylation is associated with ZO-1 rearrangement to the apical cell borders in A431 cells, a human epidermal carcinoma cell. (75) Furthermore, dephosphorylation of the tight junction proteins has been shown to precede a decrease in TER. (68) Although phosphorylation has been well documented in regulating many proteins of the TJ, this means of regulation has not been demonstrated for claudins in the epithelium. There is evidence that claudins can be phosphorylated, as claudin-5 has been shown to be phosphorylated on threonine residues in a cAMP and PKA dependent manner in endothelial cells. (34)

**Regulation of Intestinal Epithelial Tight Junctions via IFN-γ**

Interferon gamma (IFN-γ) is a pro-inflammatory glycoprotein produced primarily by T helper 1 cells and natural killer cells during inflammatory states. In vitro studies have utilized various epithelial cell lines to examine and understand the role of IFN-γ in paracellular permeability. Overall, IFN-γ has a deleterious effect on barrier function although this mechanism of action is unknown. IFN-γ significantly decreases TER in human colonic T84 cells upon basolateral administration with doses as low as 10ng/mL beginning at 24
hours after application of IFN-γ. Maximal responses occurred with 100ng/mL after 72 hours of incubation with IFN-γ.(80) The loss of TER was associated with decreased levels of ZO-1 in the NP-40 insoluble fraction, which suggested a loss of the junction-associated protein from the membrane. Loss of TER was also associated with decreased ZO-1 protein synthesis and reduced ZO-1 RNA. Of particular interest in this study was the demonstration that washout of IFN-γ after incubation for 72 hours resulted in the recovery of TER approaching baseline levels 72 hours after washout. This resolution of TER occurred despite the lack of ZO-1 in the Triton insoluble membrane fraction after washout of IFN-γ. A similar resolution of TER to baseline levels has also been reported upon washout of IFN-γ after a single 100U/mL or 1000U/mL IFN-γ dose in T84 cells.(1) These data suggest that TJ proteins other than ZO-1 might be more important in the regulation of barrier function. Madara and Stafford,(42) who observed a decrease in TER in T84 cells with doses as low as 10U/mL IFN-γ 72 hours after administration, have reported similar responses to IFN-γ. The authors did not report if IFN-γ was applied apically or basolaterally. The decrease in TER was accompanied by an increase in transepithelial flux of the paracellular probe mannitol, and was without effect on gross monolayer disruption. Basolateral application of 10U/mL IFN-γ can also reduce occludin promoter activity in a human intestinal cell line, HT-29,(46) thus showing more direct evidence that this cytokine is capable of altering expression of a specific tight junction protein. Bruewer et al.(12) have demonstrated that IFN-γ (100U/mL) decreases TER and increases the flux of the paracellular marker, FD-3, in T84 cells. This effect was supported by immunofluorescence showing redistribution of occludin, claudin-1, and claudin-4 away from the TJ. Interestingly ZO-1 maintained its localization at the TJ upon cytokine treatment. Conflicting results have been shown in CACO-2 BBE cells, an epithelial
cell line originally derived from a human colonic adenocarcinoma. In this study, IFN-γ had no effects on TER. (48) The amount of IFN-γ utilized, as well as surface application was not reported for IFN-γ.

Similar deleterious results have been observed upon application of IFN-γ in cell lines other than intestinal epithelium. For example, IFN-γ decreases TER and down-regulates claudin-1 in human thyrocytes. (55) IFN-γ also decreases TER and occludin expression in human umbilical vein endothelial (HUVEC) cells. (58)

**Regulation of Intestinal Epithelial Tight Junctions via TNF-α**

Tumor necrosis factor alpha (TNF-α) is another cytokine associated with influencing barrier function of intestinal epithelial cells. TNF-α is a proinflammatory cytokine produced mainly by macrophages, monocytes, lymphocytes and natural killer cells. Evidence exists that the proinflammatory cytokine, TNF-α has a role in intestinal disease. For example, TNF-α mRNA and protein levels are elevated in the mucosa of human patients with inflammatory bowel disease. (11,32,45,53) In vitro models utilizing CACO-2 BBE epithelial cells have demonstrated that doses as low as 5mg/mL of TNF-α can have a deleterious effect on TER with maximal effects at 100ng/mL TNF-α application to both the apical and basolateral surfaces. (48) Utilizing the human colonic cell line, HT-29/B6, there is a reduction in TER by 81% from baseline levels upon serosal addition of 100ng/mL of TNF-α for 8 hours. Additionally, there is a dose dependent response to TNF-α, with a minimum response occurring with 5ng/mL and a maximum response at 100ng/mL. Freeze fracture electron micrographs revealed that there was a reduced number of TJ strands in the presence of TNF-α.
Despite these selective effects on the tight junction, TNF-α-induced apoptosis also affects epithelial cell permeability. Rodriguez et al. used the human differentiated colon carcinoma cell line HT29 cl.19A, cloned from HT29. Cell layers were treated for 48 hours by placing 10ng/mL of TNF-α along with 5 U/mL of IFN-γ on their basolateral surface. This dose of IFN-γ was used, as it did not alter the electrical resistance at this low concentration. Utilizing freeze fracture analysis, the plasma membranes of adjacent cells appeared less fused and had less of a closed appearance in cytokine treated cells. Furthermore, cytokine treated cells, when compared to controls, had a significant decrease in the number of strands and in the depth of the TJ’s. Further data from their study revealed that short circuit current and electrical resistance also were significantly decreased in cytokine treated cells compared to controls. Finally, they reported a significant increase in flux of mannitol, sodium, and horseradish peroxidase, which further confirmed a paracellular leakage in the cell layer. Conversely, 10ng/mL of TNF-α application in T84 cells for five hours has no effect on TER. It can be speculated that the length of time for TNF-α incubation was not sufficient to induce changes in TER as the above mentioned studies utilized periods of TNF-α incubation for a minimum of 24 hours. These studies collectively point to an effect of TNF-α on paracellular permeability but they do not elucidate a potential pathway by which this cytokine disrupts the tight junction.

Cell models other than intestinal cell lines have been utilized to examine the effects of TNF-α on paracellular resistance and TJ function. In a study by Marano, et al., they have shown that TER is decreased due to the addition of TNF-α in a porcine renal epithelial cell line, LLC-PK1. The decrease in TER was accompanied by an increase in mannitol flux and
an increase in flux to other molecules such as raffinose and polyethylene glycol. TNF-α has also been shown to disrupt ZO-1 and decrease the amount of claudin-1 in MDCK cells.(59)

**IFN-γ and TNF-α Combined Effects on the TJ**

The combination of TNF-α and IFN-γ can also decrease TER and occludin promoter activity in HT-29 cells.(46) The combined treatment of these two cytokines can significantly decrease TER and increase flux of a 3 kDa paracellular solute, FD-3, in T84 cells.(12) Additionally, internalization of occludin, claudin-1, and claudin-4 were observed after 48 and 72 hours of treatment, while there were no observable effects on ZO-1. Furthermore, these effects were not due to changes in apoptosis. Interestingly, these same authors found no effects on TER with addition of TNF alone (10ng/mL TNF-α exposure for 5 hours). The combination of these two cytokines decreases TER via increased myosin light chain kinase phosphorylation.(82) Similar combined cytokine treatment studies have been performed with the mixture, Cytomix. Cytomix is a mixture of 1000U/mL INF-γ, 10ng/mL TNF-α, and 1ng/mL IL-1β. In Caco-2 cells, Cytomix increased the flux rate of FD-4, a marker utilized for determining paracellular permeability. Cytomix also decreased the amounts of occludin and ZO-1 in the Triton-X 100 insoluble fractions and caused disruption of occludin and ZO-1 at the TJ by immunofluorescence.(28) Similar results were reported by this same group in two additional publications.(27,65) Furthermore, there were no effects of Cytomix on cell death. Additionally, TNF-α and IFN-γ detrimentally affect TER in microvascular endothelial cells and fragmentation of ZO-1 via immunofluorescence.(10) The results of these studies are difficult to interpret due to the combination of these cytokines, but generally point to a selective action of these cytokines on the tight junction.
Effects of Interleukins on TJ Permeability

IL-1β can also have deleterious effects on epithelial permeability in HT29-19A cells. IL-1β (0.2-5 ng/ml) had no effect on paracellular measurements alone, but in combination with small doses (2 U/ml) of IFN-γ, did induce increases in ionic conductance and mannitol flux rates. The combination of these agents induced further decreases in epithelial barrier function, which appear to be a combined effect when visualizing the data graphically.(49) IL-1α (100U/mL, basolateral surface) decreases TER and increases inulin flux in human thyrocytes.(54) As previously reported, IFN-γ and TNF-α have a deleterious effect on barrier function in intestinal epithelium. These deleterious effects can be prevented by addition of the anti-inflammatory cytokine, IL-10. IL-10 (100ng/ml), when added to T84 cells before the addition of IFN-γ or TNF-α, is capable of attenuating increases in conductance and serosal to mucosal mannitol and inulin flux rates.(44) IL-6 is yet another cytokine shown to affect the TJ. In a mouse model of hemorrhagic shock and resuscitation, ileal mucosal ZO-1 and occludin expression is decreased in wild type mice when compared to IL-6 knockout mice. The results corresponded to the observed increase in epithelial permeability as measured by the paracellular marker FD-4.(77) Furthermore, IL-4 and IL-13 are capable of inducing decreases in TER and increased mannitol flux rates in Calu-3 cells, a human lung adenocarcinoma cell line. Interestingly, in contrast to the effects of IFN-γ in intestinal epithelium, the authors noted that 50ng/mL IFN-γ is capable of enhancing TER and preventing the deleterious effects of IL-4 and IL-13 in Calu-3 cells.(2)
Improvement of Barrier Function and the TJ

Although much attention has been focused on how the TJ is disrupted, there is a growing interest in how one can restore barrier function after insult from injury. Bryostatin-1, a nonphorbol PKC agonist, increases TER in T84 cells. The treatment of T84 cells for 4 hours with 100nM of Bryostatin-1 induced phosphorylation of occludin and a shift in claudin-1 to a Triton-X insoluble fraction, a fraction associated with the membrane.(78) Additionally, this same group of researchers has recently shown that Bryostatin-1 is capable of attenuating TNF-α induced effects on TJ permeability via PKC.(79) IL-17 is also capable of enhancing TER associated with increased expression of claudin-1 and claudin-2 mRNA in T84 cells.(37) IL-15 is yet another cytokine capable of enhancing epithelial barrier function.(69) IL-15 (100ng/ml) increases expression of claudin-1 and claudin-2 at the TJ in T84 cells and also induces phosphorylation of occludin. (55) Furthermore, it appears that IL-15 exerts its effects on the TJ via the interleukin 2-receptor β subunit. Additionally, studies utilizing ischemic injured porcine ileal mucosa have shown that PGE₂ and PGI₂ are capable of regulating the paracellular pathway. Exogenous addition of PGE₂ and/or PGI₂ is capable of improving TER and decreasing mannitol flux rates.(6-9) PGE₂ also restores occludin and ZO-1 to the area of the TJ and phosphatidylinositol-3-kinase (PI3K) appears to mediate the PGE₂ effects.(39) Interestingly, PI3K can promote adherens junction assembly in the intestinal epithelial cell line Caco-2(38) and PI3K has a pivotal role in regulating TJ integrity.(57)
Conclusions

Tight junctions are highly regulated and play a crucial role in regulating intestinal epithelial permeability to ions, nutrients and water. One of the many stimuli that may influence and control the TJ is cytokines. There is evidence that cytokines, particularly IFN-γ and TNF-α, influence TJ function and increase paracellular permeability in intestinal epithelium. Additionally, there are other mediators of TJ function including additional interleukins, such as IL-17, prostaglandins, and PKC agonists, which are capable of enhancing barrier function. Thus, based upon the data, it was apparent that there was a lack of evidence for understanding the mechanisms by which TJ’s can be regulated. We sought to determine the mechanism by which PGE₂ is capable of regulating TJ function and enhancing barrier function. Insight into the mechanisms by which PGE₂ can regulate TJ structure and function could lead to novel treatment options for various intestinal disease states.
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CHAPTER TWO

THE REGULATION OF CYCLOOXYGENASE-2 EXPRESSION AND ITS ROLE IN
RECOVERY OF INTESTINAL BARRIER FUNCTION
Introduction

Intestinal mucosal inflammation, such as occurs during inflammatory bowel disease, often results in an influx of neutrophils into the mucosa. The function of the neutrophil is the capture and destruction of foreign organisms and proteins through phagocytosis. Neutrophils are considered a first line of defense against invading organisms and are a vital component of innate immunity, which requires no prior exposure to these organisms for activation. However, prolonged or excessive activation of neutrophils may be deleterious due to release of toxic mediators such as reactive oxygen metabolites. Furthermore, neutrophils are an important source of eicosanoids, in particular leukotriene B4 (LTB$_4$), thromboxane A2 (TXA$_2$), and prostaglandin E$_2$ (PGE$_2$) elaborated by cyclooxygenase (COX) isoforms such as COX-2.(18,22,69) Additionally, neutrophils are associated with elaboration of cytokines, such as IL-1$\beta$, which can regulate cyclooxygenase-2 (COX-2). The expression of COX-2 and elaboration of PGE$_2$ are important for recovery of barrier function in ischemic injured porcine ileum(5-7,57,58). Additionally, mice lacking the COX-2 isoform have impaired intestinal mucosal defense to dextran sodium sulfate induced colitis, thus further demonstrating the importance of COX-2 in intestinal health.(44) Evidence also exists for prostaglandins having a cytoprotective role in the gut(53,54) and stimulating reparative mechanisms in injured gastrointestinal epithelium.(15) Furthermore, PGE$_2$ regulates gastric mucosal inflammation and contribute to mucosal integrity during H. Pylori infection in mice.(60) The regulation of COX-2 and elaboration of PGE$_2$ could thus have important implications in various clinical situations involving inflammation. Likely candidates for regulation of COX-2 are the mitogen activated protein kinase (MAPK) pathways as this family of kinases has been shown to be involved in regulating many physiological processes,
including inflammatory conditions. For example, in Crohn’s disease, in vivo reductions in c-Jun NH2 terminal kinase (JNK) activity results in endoscopic evidence of intestinal healing and clinical improvement in these patients. (29) Thus, the regulation of COX-2 and expression of PGE$_2$ via the MAPK pathway could have important clinical implications during inflammatory states.

**Neutrophil expression of COX-2**

COX-2, also known as prostaglandin G/H synthase-2, is one of the primary rate-limiting COX enzymes in the biosynthesis of prostaglandins from arachidonic acid. Neutrophils are one cell type, which can express and regulate COX-2. Lipopolysaccharide (LPS)-stimulated human neutrophils are capable of expressing COX-2 (38) and this gene expression also results in elaboration of PGE$_2$. (17, 47) Murine peritoneal neutrophils, harvested after exposure to burn injury, express increased COX-2 and PGE$_2$ levels at four hours after injury. (27) Conversely, in primates with acute severe pneumonia, neutrophils in inflammatory lung lesions do not express COX-2. (33)

**Interleukin-1 regulation of COX-2**

Interleukin-1 (IL-1) is a pro-inflammatory cytokine that may be elaborated by neutrophils and is associated with modulating the inflammatory response. (14) Elevated levels of IL-1 have been consistently observed in tissues from humans and animals with a variety of inflammatory bowel diseases. (1, 9, 40, 66) IL-1 elevates COX-2 protein levels in human intestinal myofibroblasts. (13, 41) Similar results have been demonstrated in primary subepithelial fibroblast strains isolated from human colonic biopsies. IL-1β stimulation of
these cells for 24 hours resulted in upregulation of COX-2 mRNA and protein levels. (68) Furthermore, treatment with IL-1 (30ng/mL) in Mode K cells, a murine intestinal epithelial cell line, is capable of increasing COX-2 levels at one hour after IL-1 administration. (28) Additionally, upregulation of COX-2 mRNA and protein occurs in HT-29 and Caco-2 colonic epithelial cells. (64) IL-1β (10ng/mL) is also capable of rapidly inducing COX-2 protein expression during recovery of porcine ischemic injured ileal mucosa. (57) Collectively, these studies demonstrate that interleukin-1 is capable of inducing COX-2 levels in intestinal epithelium.

Similarly, IL-1-induced COX-2 responses occurs in non-intestinal cells. IL-1β elevates COX-2 protein levels and PGE₂ levels in cerebral blood vessels of rats. (49) Furthermore, IL-1β is capable of regulating COX-2 in human airway smooth muscle cells, (46) human myometrial cells, (59) and human gingival fibroblasts. (62)

**Additional cell types and modulators of COX-2**

Additional cell types are also capable of expressing COX-2 and PGE₂. In human intestinal smooth muscle cells eight hours of exposure to LPS (100µg/mL) or IL-1β (100 units/mL) results in increased COX-2 protein expression and PGE₂ production. (37) Similar responses have also been observed in human enterocytes. (36) LPS exposure for three hours in bovine aortic smooth muscle cells also results in increased COX-2 mRNA and protein expression. (56) Furthermore, in dogs with glaucoma, there is an increase in COX-2 expression in all corneal layers with the greatest COX-2 expression in corneal epithelium. (39) Thus, it is evident that multiple cells are capable of expressing COX-2 when stimulated.
Mitogen Activated Protein Kinases

MAPK’s are a conserved family of enzymes that form a highly integrated network controlling various cellular functions such as cell growth, differentiation, proliferation, and cell death.(10) The MAPK pathways transduce signals from a diverse array of extracellular stimuli. The three primary MAPK signaling pathways (Fig.1) are the extracellular regulated kinases (ERK 1&2), p38 MAPK, and c-Jun NH₂-terminal kinase (JNK).(10) Phosphorylation of threonine and tyrosine residues is essential for activation of each of the three primary MAPK’s and this is achieved by MAPK-kinases. Once activated the MAPK’s regulate cellular events including phosphorylation of kinases and cytoskeletal elements.(8) MAPK’s may also translocate to the nucleus to phosphorylate transcription factors (TF) such as c-Jun, c-Fos, and Elk-1.(25,26,32,63) MAPK’s can also regulate TF function by controlling the amount of the TF by either modulating transcription factor expression levels or the stability of the proteins. An example of how phosphorylation of TF’s can affect their stability has been demonstrated via the JNK pathway. The association of inactive JNK with its TF target such as cJun or ATF-2 is reported to target them for ubiquitination and degradation. Activated JNK phosphorylates these TF’s, which protects them from degradation and therefore contributes to promoting transcriptional activation.(20,21,45) While ERK 1&2 are primarily activated by hormones and growth factors, p38 MAPK and JNK are principally activated by cytokines and environmental stresses.(31,34) Thus, the stress activated MAPK’s, p38 MAPK and JNK, appear to be likely candidates for regulating COX-2 during periods of inflammation.
MAPK regulation of COX-2

MAPK’s are one group of candidate molecules for regulation of COX-2 since they have been shown to be involved in the signal transduction pathways leading to pro-inflammatory gene transcription in inflammatory states. (3,19) MAPK regulation of COX-2 has been demonstrated in many cell culture models utilizing various stimuli. Osmolarity induced COX-2 can be suppressed with p38 MAPK inhibition in colonic epithelial cells. (2) Furthermore, basic fibroblast growth factor induced COX-2 in human intestinal epithelial cells is also positively regulated by p38 MAPK. (61) Additionally, in rat intestinal epithelial cells, gastrin stimulates activation of all three of the primary MAPK’s and each of the three primary MAPK’s is required for gastrin induced COX-2 expression. (23) Furthermore, in rat intestinal epithelial cells, bombesin (100nM), a homolog of gastrin-releasing peptide,
stimulates increased COX-2 mRNA levels after one hour of treatment with maximal effects at two hours. Correspondingly, COX-2 protein levels are also elevated in rat intestinal epithelial cells, which can be observed via Western Blot analysis at one hour with a maximal effect at six hours. Additionally, there was an increase in PGE$_2$ levels in the bombesin-treated cells. Finally, these authors showed that the ERK 1&2 inhibitor, PD-98059 (10µM) and the p38 MAPK inhibitor, SB-203580 (10µM) could each inhibit the increase in bombesin-induced COX-2 mRNA levels but neither inhibitor was capable of abolishing the COX-2 mRNA increase alone. (24) Interestingly, in HT-29 cells, eight hours of treatment with the non-steroidal anti-inflammatory drug (NSAID) flufenamic acid induced COX-2 protein expression in a dose dependent manner (10, 100, and 200µM). Similarly, eight hours of treatment with the NSAID, sulindac sulfide, also increased COX-2 protein expression in a dose dependent manner (10, 50, and 100µM). Inhibiting p38 MAPK or ERK 1&2 altered neither the flufenamic acid nor the sulindac sulfide-induced increases in COX-2 expression. (50) Thus, it appears that these two NSAID’s are capable of increasing COX-2 expression via MAPK independent pathways. In human monocytes, COX-2 expression requires the activation of p38 MAPK. (48,51) Additionally, heat shock protein 60 is yet another stimulus capable of inducing COX-2 expression in rat endothelial cells and alveolar macrophages via an ERK1&2 and p38 MAPK dependent pathway. (4) Our laboratory has demonstrated that p38 MAPK positively regulates COX-2 protein expression in ischemic-injured porcine ileum while JNK negatively regulates COX-2. Furthermore, ERK 1&2 does not appear to regulate COX-2 in this model. (58)

MAPK’s are also capable of regulating IL-1-induced COX-2 expression. Utilizing intestinal myofibroblasts, Mifflin et al. (41) have demonstrated that IL-1α (500pg/mL)
induces COX-2 RNA and protein levels at one and four hours, respectively. Furthermore, ERK inhibition results in an approximate 78% reduction in IL-1α-induced COX-2 protein induction while p38 MAPK inhibition results in an approximate 93% reduction in IL-1α-induced COX-2 protein levels. In HT-29 human colon cancer cells, IL-1β increased COX-2 mRNA and protein expression and inhibitors of the ERK 1&2, JNK, or p38 MAPK pathways were capable of preventing the IL-1β induced expression of COX-2. (35) Confounding the issue are data showing that IL-1β-induced upregulation of COX-2 is mediated through the JNK pathway in rat mesangial cells (55) whereas in pulmonary epithelial cells (65) and macrophages. (43) IL-1β-induced upregulation of COX-2 is not mediated through the JNK pathway. Additionally, there is possible cross-communication among the MAPK pathway despite the three major components being viewed as parallel. In human umbilical vein endothelial cells, IL-1α activated ERK1&2 and induced increases in PGI₂ production while administration of the p38 MAPK inhibitor, SB-203580, resulted in an enhancement of IL-1α-induced ERK 1&2 phosphorylation. (30)

An interesting aspect of the p38 MAPK pathway is the mechanism by which this kinase is capable of regulating COX-2 expression. It has been proposed that p38 MAPK regulates COX-2 by increasing mRNA stability. Treatment of LPS-stimulated monocytes with a p38 MAPK inhibitor results in rapid degradation of COX-2 mRNA. (11) Furthermore, IL-1 is capable of inducing COX-2 protein expression and PGE₂ production in HeLa cells and that p38 MAPK regulates COX-2 mRNA stability in this model as well. (52) Additionally, chenodeoxycholate, a bile acid promoter of gastrointestinal cancer, results in p38 MAPK phosphorylation and stabilizes COX-2 mRNA. In addition, expression of a dominant negative JNK had no effect on COX-2 mRNA stability with chenodeoxycholate.
treatment. In human intestinal myofibroblasts aspirin paradoxically results in prolonged increases in COX-2 expression. This effect was a result of activation of p38 MAPK and phosphorylation of this kinase resulting in stabilization of COX-2 mRNA.

In human synovial fibroblasts, IL-1β induces a sharp (<30 fold) and immediate (5 min) rise in COX-2 mRNA. Additionally, there was an upregulation of COX-2 protein and the effects of IL-1β on mRNA and protein levels were a result of p38 MAPK activation, which stabilized COX-2 mRNA levels.

Furthermore, the mechanism by which p38 MAPK acts to regulate COX-2 mRNA stability has been discovered. Previously, the mechanism by which the MAPK cascade, in particular p38 MAPK, is capable of regulating COX-2 expression was unknown. It has been shown that p38 MAPK regulates mRNA stability by regulating deadenylation.

Conclusion

Based upon reported data, it appears that multiple cells types are capable of expressing COX-2 under a variety of stimuli. Furthermore, the MAPK pathway appears to be one intermediate step in regulating COX-2 expression. The determination of which MAPK is regulating COX-2 depends upon not only the cell type, but also the stimulus. Much attention has been focused on the p38 MAPK as it appears to be activated under a variety of stress conditions. As mentioned previously, p38 MAPK is capable of regulating COX-2 via mRNA stabilization. Data regarding ERK 1&2 regulation of COX-2 is not uniform. In some instances ERK 1&2 appears to positively regulate COX-2 expression while in other instances ERK 1&2 has no effect on COX-2 expression. The regulation of COX-2 by JNK has not been as thoroughly examined as p38 MAPK and ERK 1&2. Based upon the limited data available, it appears that JNK has a negative regulatory effect on COX-2 and inflammation.
As a result of the conflicting data, we sought to determine the mechanisms regulating COX-2 expression in porcine ischemic injured ileum. We wanted to determine if neutrophil and/or IL-1β could induce expression of COX-2 in our model. Furthermore, we examined the role of the MAPK cascade in regulating COX-2 expression. The regulation of COX-2 could have meaningful implications as COX-2 expression leads to elaboration of PGE$_2$, which has been shown to enhance intestinal barrier function.
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NEUTROPHILS AUGMENT RECOVERY OF PORCINE ISCHEMIA-INJURED ILEAL MUCOSA BY AN IL-1β AND COX-2 DEPENDENT MECHANISM

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Short Title: Neutrophils augment recovery of ischemia-injured ileum

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Abstract
Shifflett, Donnie E., Frank G. Bottone Jr., Karen M. Young, Adam J. Moeser, Samuel L. Jones, and Anthony T. Blikslager. Neutrophils augment recovery of porcine ischemia-injured ileal mucosa by an IL-1β and COX-2 dependent mechanism. - Neutrophils (PMNs) play a critical role in intestinal mucosal injury and repair. To study the effects of PMNs on acutely injured mucosa, we applied PMNs isolated from circulation or peritoneal fluid from animals with chemically-induced peritonitis to ischemic-injured porcine ileal mucosa. In preliminary experiments, PMNs enhanced recovery of transepithelial electrical resistance (TER), and this action was inhibited by pre-treatment with the non-selective COX inhibitor indomethacin. Because COX-2 is up-regulated by inflammatory mediators such as IL-1β, which is released by PMNs, we postulated that PMNs enhance recovery of ischemia-injured mucosa by a pathway involving IL-1β, and COX-2. Application of 5x10⁶ PMNs to the serosal surface of ischemia-injured mucosa significantly enhanced recovery of TER (p<0.05), an effect that was inhibited by the selective COX-2 inhibitor NS-398 (5µM), and by an IL-1β receptor antagonist (0.1mg/ml). Addition of 10ng/mL IL-1β to the serosal surface of injured tissues caused a significant increase in TER (p<0.05), which was inhibited by pre-treatment with NS-398. Western blots of mucosal homogenates revealed dramatic up-regulation of COX-2 in response to IL-1β or peritoneal PMNs, and the latter was inhibited by an IL-1β receptor antagonist. Real time PCR revealed increased mRNA COX-2 expression preceded increased COX-2 protein expression in response to IL-1β. We concluded that PMNs augment recovery of TER in ischemia-injured ileal mucosa via IL-1β-dependent upregulation of COX-2.

Key Words: intestine, prostaglandin, transepithelial electrical resistance, tight junction
Introduction

Polymorphonuclear neutrophils (PMNs) are a critical component of the inflammatory response associated with a variety of gastrointestinal pathophysiological conditions, including microbial invasion (18) and ischemia/reperfusion injury (25). Once PMNs have reached sites of tissue invasion or injury, they release a number of inflammatory mediators which may exacerbate injury, but which are also vital to host defense. These neutrophilic mediators include reactive oxygen metabolites, proteases, and cytokines such as interleukin-1β (IL-1β).

IL-1β is a pro-inflammatory cytokine whose primary role is to modulate and amplify inflammatory responses (13). Previous studies have shown that PMNs, upon exposure to either LPS or granulocyte-macrophage CSF, release IL-1β (1, 7, 42). Furthermore, elevated levels of IL-1β have been consistently observed in tissues from humans and animals with a variety of inflammatory bowel diseases (1, 10, 30, 31, 44). IL-1β appears to mediate its inflammatory effects via the cyclooxygenase (COX) (21, 22, 32, 39, 41) and mitogen activated protein kinase (MAPK) pathways (14–15). However, the precise effects of the IL-1 family of cytokines on tissues are variable. For example, IL-1 has been shown to be harmful to cultured intestinal epithelial monolayers (29), but when IL-1 was added to T84 cells in the presence of subepithelial co-cultured myofibroblasts, epithelial secretion was the most notable effect associated with upregulation of COX-2 (21, 32).

At least three isoforms of COX exist: COX-1, which is constitutively expressed in many tissues, an inducible COX-2 isoform (36), and COX-3, which is a COX-1 variant (11). COX-2 can be upregulated by a number of stimuli including growth factors and cytokines (20, 35) and appears to play an important role in recovery of injured intestinal mucosa (5,
For example, COX-2 was expressed in epithelium at the margins of experimentally induced gastric ulcers in rats, and selective inhibition of COX-2 retarded epithelial repair (33). Alternatively, while inhibition of COX-2 did not interrupt recovery of barrier function in ischemia-injured mucosa in previous studies from our laboratory, COX-2 prostanoids were able to stimulate recovery in the absence of COX-1 prostanoids (5).

We have recently shown that PMNs migrate across restituting epithelium during the inflammatory phase of epithelial wound healing, thereby disrupting recovery of mucosal barrier function (17). However, in preliminary studies in which PMNs harvested from circulation were incubated with acutely injured mucosa, we noticed an enhancement rather than disruption of the recovery process that was sensitive to the non-selective COX inhibitor indomethacin. We hypothesized that this effect resulted from up-regulation of COX-2, and we sought to determine the critical elements of the signaling pathway that led to PMN-enhanced recovery of transepithelial electrical resistance (TER) in ischemia-injured porcine ileum.
Methods and Materials

Experimental surgery

All procedures were approved by the North Carolina State University Institutional Animal Care and Use Committee, and have been previously described in detail (3, 4). Briefly, 6-8 week-old Yorkshire-cross pigs of either sex were anesthetized using a combination of xylazine (1.5mg/kg) and ketamine (11mg/kg). Pigs were intubated via a tracheostomy, placed on a heating pad and ventilated with 100% O₂ via a tracheotomy using a time-cycled ventilator. Anesthesia was maintained using periodic IV administration of a 5% sodium thiopental solution via a jugular catheter. Maintenance fluids were administered at a rate of 15ml/kg/min throughout the surgery. A carotid cutdown and catheterization was performed for collection of blood for harvesting of PMNs. The ileum was located via a midline incision, after which 10-cm ileal segments were ligated and subjected to ischemia by ligating the local mesenteric blood supply. Additional 10-cm ileal loops not subjected to ischemia were utilized as control tissues. After 45-min ischemia, pigs were euthanized with an overdose of pentobarbital and 10-cm ileal loops were promptly removed and placed in oxygenated Ringers solution (95% O₂/5% CO₂).

Ussing chamber studies

The mucosa was stripped from the seromuscular layer and mounted in 3.14 cm² aperture Ussing chambers. The tissues were bathed on both serosal and mucosal sides with 10ml of oxygenated (95% O₂/5% CO₂) Ringer’s solution. In addition, the serosal bathing solution contained 10mM of glucose, which was osmotically balanced by 10mM mannitol on the mucosal side. Bathing solutions were circulated in water-jacketed reservoirs, and maintained at 37°C. The spontaneous potential difference (PD) was measured using Ringer-
agar bridges connected to calomel electrodes, and the PD was short-circuited through Ag-AgCl electrodes using a voltage clamp that corrected for fluid resistance. Resistance (Ω·cm²) was calculated from the spontaneous PD and short-circuit current (Isc). If the spontaneous PD was between -1.0 and 1.0 mV, tissues were current clamped at ±100 μA for 5 seconds and the PD recorded. Short-circuit current and PD were recorded every 15-min for 180-min.

**Blood PMN collection**

Arterial blood (20 ml) was collected from the carotid catheter, heparinized, and thoroughly mixed with 6% dextran (4 ml). Red blood cells were allowed to settle for 60 min. The leukocyte rich plasma was drawn off and overlayed onto 5ml of Ficoll-Paque and centrifuged at 1800xG for 20 min. The plasma and Ficoll were removed and the remaining pellet was suspended in 40 ml HBSS and counted using a hemacytometer. Cell viability was determined using Trypan Blue. Viability was consistently > 98%. The suspended cells were then centrifuged at 1000xG for 10 min. The HBSS was drawn off and the cells resuspended in Ringers solution for use. Neutrophil purity using this method was > 97% (data not shown). PMNs (1 or 5x10⁶ total cells) were applied to the serosal side of the tissue 45-min after equilibration of the tissues on the Ussing chamber.

**Extravasated PMN collection**

Peritonitis was induced in pigs for collection of extravasated PMNs. Briefly, 6-8 week-old Yorkshire-cross pigs of either sex were anesthetized using a combination of xylazine (1.5mg/kg IM) and ketamine (11mg/kg IM). Buprenorphine was administered (0.05mg/kg IM) for analgesia. Subsequently, 15-20 mL of thioglycollate was injected into the peritoneal cavity. Each pig was then allowed to recover for 4 hours after which they were
again anesthetized using a combination of xylazine (1.5mg/kg IM) and ketamine (11mg/kg IM). Pigs were then euthanized with an overdose of pentobarbital and a midline incision was performed to allow entry to the peritoneal cavity. Fluid was collected from the peritoneum. Collected cells were spun down and the pellet was resuspended in 20 ml HBSS and counted using a hemacytometer. Cell viability was determined using Trypan Blue. Viability was consistently > 98%. The suspended cells were then centrifuged at 1000xG for 10 min. The HBSS was drawn off and the cells resuspended in Ringers solution for use.

**Experimental Treatments**

Recombinant porcine IL-1β and recombinant porcine IL-1β receptor antagonist were purchased from R&D Systems (Minneapolis, MN). NS-398 was purchased from Caymen Chemical (Ann Arbor, MI). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

**Gel Electrophoresis and Western Blotting**

Tissues mounted in the Ussing chambers were removed at 75 min. after the initiation of the experiment. Mucosal scraping was performed in order to remove additional connective tissue, after which tissue was snap-frozen in liquid nitrogen and stored at −70°C until analysis for COX 1 & 2 proteins. In preparation for SDS-PAGE, tissues were thawed to 4°C. One-gram tissue portions were added to 3ml of chilled RIPA buffer (0.15M NaCL, 50 mM Tris [pH 7.2], 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS), including protease inhibitors (PMSF and aprotinin). The mixture was homogenized on ice and then centrifuged twice at 10,000g for 10 min at 4°C and the supernatant saved. Protein analysis of extract aliquots was performed (DC protein assay, Bio-Rad, Hercules, CA). Tissue extracts (amounts
equalized by protein concentration) were mixed with an equal volume of 2X- SDS-PAGE sample buffer and boiled for 4 min at 100°C. Lysates were loaded on a 10% SDS-polyacrylamide gel and electrophoresis was carried out according to standard protocols. Proteins were transferred to a nitrocellulose membrane (Hybond ECL, Amersham Life Science, Birmingham, UK) using an electroblotting mini-transfer apparatus according to the manufacturer’s protocol. Membranes were blocked overnight at 4°C in Tris buffered saline (TBS) and 5% dry powered milk. Membranes were washed two times with TBS containing 0.05% Tween (TBS-T) and incubated for 2 hours at room temperature in primary antibody (COX-1 or COX-2, affinity-purified goat polyclonal antibodies, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After washing three times for 5 min each with TBS-T, the membranes were incubated for one hour with horseradish peroxidase conjugated secondary antibody. After washing two times for 5 min each with TBS-T and one time with TBS for 15 min, the membranes were developed for visualization of protein by addition of enhanced chemiluminescence reagent (Amersham, Princeton NJ).

**mRNA Analysis**

*Reverse Transcription.* RNA was treated with 1 unit (U) of amplification grade Deoxyribonuclease I (Life Technologies, Gaithersburg, MD) per µg of RNA at room temperature for 15 min to remove genomic DNA followed by inactivation of the Deoxyribonuclease I with 2.5 mM EDTA (pH 8.0). The RNA was then incubated at 65°C for 5 min followed by quantitation. Two µg of the RNA was reverse transcribed using 100 units of Superscript-II reverse transcriptase according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). After RT, cDNA was treated with 1U RNase H (Life
Technologies, Gaithersburg, MD) per µg RNA at 37°C for 20 min. RT was performed using Qiagen’s Omniscript reverse transcription kit according to the manufacturer’s instructions. A negative control containing all of the RT reagents in the absence of RT enzyme (no RT control) was also routinely performed. The cDNA was diluted 5X with RNase-free water (Ambion, Austin, TX) and stored at -80°C until use.

**Primer Design.** Primers were designed using PrimerExpress Software (Applied Biosystems, Foster City, CA) from known pig (*Sus scrofa*) sequences found in Genbank™. Primers were from Life Technologies and dissolved in 10 mM Tris, pH 7.0. Primers are listed as follows: accession number (common name): forward primer; reverse primer (product size). U07786 (Actin): CTCCTTCTGGGGCATCGGA; CGCACTTCATGATCGAGTTA (65). AY028583 (prostaglandin G/H synthase-2, PGHS-2): TGTATCCTCGACAGCCAAAG; GCGGAGGTGTTCAGGAGTGT (71).

**Real-time RT-PCR with Sybergreen Detection.** Traditional and Real-time RT-PCR were performed using an ABI Prism 7700 (Applied Biosystems, Foster City, CA) as previously described (8). Real-time RT-PCR fluorescence detection was performed in 96-well plates using Quantitect SYBR Green buffer (Qiagen). Each 50 µl PCR reaction contained cDNA, 0.5U of Amp Erase Uracil-N-glycosylase (UNG) (Perkin Elmer Life Sciences, Boston, MA), forward and reverse primers, and the Passive Reference dye (ROX) to normalize the SYBR green/double stranded DNA complex signal during analysis to correct for well-to-well variations while primer concentrations were optimized to yield the lowest concentration of primers that yielded the same Ct values as recommended by Applied Biosystems. A no RT control RNA sample was used with each real-time RT-PCR experiment containing human actin primers to verify no genomic DNA contamination.
Amplification parameters were UNG incubation, for one cycle at 50°C for 2 minutes to prevent amplification of carryover DNA; denaturation/UNG inactivation at 94°C for 10 min; amplification, 40 cycles of 95°C/15 sec and 60°C/60 sec. Amplification products using Sybergreen detection were checked using dissociation curve software (Perkin Elmer) and by gel electrophoresis on a 1% agarose gel then visualized under UV light following staining with 0.05% ethidium bromide to confirm the size of the DNA fragment, and that only one product was formed. Samples were compared using the relative (comparative) Ct method. The Ct value, which is inversely proportional to the initial template copy number, is the calculated cycle number where the fluorescence signal emitted is significantly above background levels. Fold induction by real-time RT-PCR was measured in triplicate wells to account for RT-PCR and repeated with 2 animals and calculated after adjusting for actin using $2^{-\Delta\Delta Ct}$, where $\Delta Ct = target \ gene \ Ct - actin \ Ct$, and $\Delta\Delta Ct = \Delta Ct \ control - \Delta Ct \ treatment$.

**Data Analysis**

All data were analyzed using a statistical software package (Sigmastat, Jandel Scientific, San Rafael, CA). Data were reported as mean ± standard error of the mean (SE) for a given number (‘n’) of animals for each experiment. All treatments for each experiment were applied to tissues from each of the animals, but there were no duplicate treatments on any of the animals. Electrical data were analyzed by repeated measures 2-way ANOVA for the effects of time and treatment on TER, using the animal number as the subject. For a significant result on the initial 2-way ANOVA for each set of data, a Tukey’s test was utilized for pairwise multiple comparisons to discern differences between treatments. P values reported for each experiment on electrical data represent the result of the repeated measures ANOVA for the time period over which treatments were applied, rather than the
differences between treatments at a single time point. For data on IL-1\(\beta\) levels, a 2-way
ANOVA for the effects of time and treatment was utilized, followed by a post-hoc Tukey’s
test for pairwise comparisons. The \(\alpha\) level for statistical significance was set at \(p<0.05\).
Results

Neutrophils increase transepithelial electrical resistance

Because of our interest in the effects of PMNs on recovery of acutely injured mucosa, we initially applied either 1 or 5x10^6 PMNs harvested from arterial blood to the mucosal or serosal surface of ischemia-injured ileum from the same animal following an initial 45-min equilibration period. The number of PMNs for application was based upon the results of work by Gayle et. al showing peak PMN infiltration of 700 PMNs/mm^2 in porcine post-ischemic ileal mucosa (17). Mathematical calculations utilizing the surface area of the tissue exposed to PMNs within Ussing chambers (3.14cm^2) and the effect of villus surface area amplification (3), indicated that 1-10 million PMNs per chamber would approximate this peak number of PMNs. These PMNs were allowed to interact with the tissue within Ussing chambers for 15-min prior to circulating 10ml of Ringers solution through the chambers from the mucosal and serosal fluid reservoirs. Although there was no significant effect of low numbers of PMNs (1x10^6 cells), there was a sustained increase in TER following addition of 5x10^6 PMNs to the serosal surface of tissues (Fig. 1). Conversely, there was no significant effect of PMNs applied to the mucosal surface of tissues, and there was no effect of PMNs on normal tissues (data not shown). Orienting the chambers vertically in an attempt to facilitate PMN contact with tissues did not alter the magnitude of change in TER following serosal application of PMNs. Microscopic evaluation of tissues did not reveal a significant increase in the number of adherent PMNs compared to tissues in the absence of added PMNs (data not shown). Additionally, there was no effect of pre-treatment of PMNs with the anti-β2 integrin monoclonal antibodies IB4 or R15.7 (40µg/ml each, data not shown). Taken together, these
data suggested that PMNs enhanced recovery of TER when applied to the serosal surface of ischemia-injured tissues by a mechanism independent of PMN infiltration of the tissue.

**Neutrophil effects on ischemia-injured ileum are COX-2 and IL-1β-dependent**

Since we have previously shown an important role of prostaglandins in orchestrating recovery of TER in ischemia-injured porcine ileum (4), tissues were pre-treated with the non-selective COX inhibitor indomethacin (5µM) or the selective COX-2 inhibitor NS-398 (5µM) followed by serosal application of 5x10^6 PMNs harvested from whole blood after an initial 45-min equilibration period. Indomethacin inhibited both baseline recovery of TER and PMN-stimulated recovery of TER (Fig. 1), whereas NS-398 inhibited the PMN-induced increases in TER, which were observed upon addition of PMNs alone, but had no effect on baseline recovery of TER (Fig. 2). We next considered potential PMN mediators that might be responsible for stimulating COX-2. Although there was no effect of pre-treatment of tissues with the anti-oxidant catalase (1000 units/ml), pre-treatment of tissues with 0.1 mg/ml of an IL-1β receptor antagonist prevented the PMN-induced rise in TER (Fig. 2). Analysis of fluid for IL-1β showed that PMNs suspended in Ringers (5x10^6 PMNs/ml) prior to addition to tissues in the Ussing chamber contained ~100ng/ml IL-1β, whereas fluid bathing ischemia-injured tissues prior to PMN addition contained ~20ng/ml. After 180-minutes of in vitro recovery, tissues exposed to PMNs had significantly greater levels of IL-1β than untreated tissues (Fig. 3). Collectively, these data indicated that the pathway utilized by PMNs to stimulate increases in TER involved both IL-1β and COX-2.
**IL-1β dose response**

To determine if IL-1β addition could improve TER in the absence of PMNs, and to determine if this effect was dose-dependent, IL-1β was added to the serosal side of ischemia-injured porcine ileal mucosa after an initial 45-minute equilibration period. As shown in Fig. 4, IL-1β at a dose of 10ng/ml had a significant effect (p<0.05) on improving TER when compared to untreated tissue, whereas lower or higher doses (1ng/ml and 100ng/ml respectively) were without effect. The specific dose response improvement in TER observed was only noted upon addition of IL-1β to the serosal side of tissue, as there was no effect when IL-1β was applied to the mucosal side of tissue (data not shown). IL-1β was also applied at the same doses to uninjured tissue, but had no effect on TER when applied to the serosal side of control tissue (data not shown). Based upon this information, it appears that IL-1β interacts with subepithelial tissues primed by ischemia to induce early recovery of TER.

**The role of COX-2 in the IL-1β-induced increases in TER**

We next determined if COX-2 played a role in the increased TER seen with 10ng/ml IL-1β by pre-treating tissues with the selective COX-2 inhibitor NS-398 (5µM). Similar to the effect of NS-398 on tissues exposed to PMNs, pre-treatment of ischemia-injured tissues with NS-398 blocked the increase in TER that was observed upon serosal addition of 10ng/ml of IL-1β after an initial 45-minute equilibration period (Fig. 5). To further investigate the relationship between IL-1β and COX isoforms, Western blot analysis of IL-1β-treated ischemia-injured tissues was performed. Tissues from 6 animals were removed from the Ussing chambers after addition of 10 ng/ml of IL-1β at the earliest time point at
which increases in TER were noted (15-min following IL-1β addition, and 60-minutes following initiation of the experiment). The same tissue was also subjected to Western blot analysis for COX-1 protein. As seen in Fig. 6, IL-1β at 10ng/ml appreciably upregulated COX-2 when compared to untreated ischemia-injured tissue, whereas there was no notable effect on COX-1 expression (not shown). On additional blots not shown, COX-2 could be appreciated in untreated ischemia-injured tissue lanes, but IL-1β-treated resulted in marked up-regulation of COX-2. Due to the rapid upregulation of COX-2 in IL-1β treated mucosa, mRNA analysis was performed at 5, 10, and 15 min following IL-1β treatment. Fig. 7 shows that COX-2 mRNA is elevated in IL-1β-treated ischemia-injured tissue when compared to untreated ischemia-injured tissue at 5 min. However, expression levels were similar in both sets of tissues at 10-minutes, and by 15 min, COX-2 mRNA expression was decreased when compared to untreated ischemic injured mucosa, suggesting that mRNA had largely been transcribed and degraded at this point, giving rise to increased protein expression. Overall, RT-PCR and Western analyses supported the hypothesis that IL-1β upregulates COX-2. Furthermore, the blockade of IL-1β-induced increases in TER with the COX-2 inhibitor NS-398 indicates that increased COX-2 expression is responsible for enhanced recovery of ischemia-injured mucosa.

**Extravasated PMNs enhance recovery of TER**

In further experiments, we wished to study the effects of extravasated PMNs in order to determine if post-circulatory PMNs would have a similar effect on recovery of TER in ischemic-injured mucosa. Extravasated PMNs were harvested from peritoneal fluid induced in a separate pig. A separate pig was utilized for the induction of peritonitis to ensure no
effect of peritoneal PMNs infiltration on experimental intestine. These extravasated PMNs were considered to be a physiologically relevant population of cells, since PMNs migrating in repairing mucosa in vivo would have already exited the microcirculation. As shown in Fig. 8, extravasted PMNs had a similar effect on TER when applied at a dose of $5 \times 10^6$ cells on the serosal side after an initial 45-minute equilibration period.

The role of COX-2 in the PMN-induced increases in TER

To further investigate the hypothesis that the improvement in TER upon addition of $5 \times 10^6$ PMN was due to an increase in COX-2 protein expression that was IL-1β dependent, tissues equilibrated for 45-minutes were exposed to extravasated PMNs for 30-minutes in the presence or absence of an IL-1β receptor antagonist added at the beginning of the equilibration period, and Western blotted for COX-2. Mucosal homogenates from untreated, ischemic-injured ileum revealed notable expression of COX-2, whereas mucosal homogenates from tissues exposed to extravasated PMNs had dramatically increased expression of COX-2 (Fig. 9). Furthermore, the increase in COX-2 protein in PMN-treated tissue was prevented by pre-treatment of tissues with an IL-1β receptor antagonist. Thus, this data supports the hypothesis that PMNs are capable of improving TER in ischemic injured tissue via an IL-1β and COX-2 dependent pathway.
Discussion

PMNs are attracted to sites of injury, during which time they are activated, and may exacerbate injury as a result of release of proteases, oxidants, or pro-inflammatory cytokines (16, 25, 37, 38). However, PMNs are a critical component of innate immunity that also appears to have a beneficial role when applied to acutely-injured porcine ileal mucosa. Specifically, addition of $5 \times 10^6$ PMNs hastens recovery of TER via an IL-1β and COX-2 dependent mechanism. We do not know the physiologic relevance of these findings, since PMN infiltration into acutely injured mucosa is not immediate following ischemic injury. For example, peak numbers of infiltrating PMNs recorded in our previous studies occurred at 6-hours following the initial ischemic event (17). However, there are resident PMNs in mucosa that would become activated in the early phases of the injury-repair cycle (26) that could presumably release IL-1β in the immediate vicinity of repairing epithelium. Furthermore, the beneficial effects of neutrophilic IL-1β release would be counter-balanced by PMN transmigration across repairing epithelium, which we have previously shown is capable of retarding the mucosal recovery response (17), although this effect is dependent on the number of transmigrating PMNs (34). Thus the net effect of PMNs on repairing epithelium would relate to factors such as the number of PMNs activated within the vicinity of repairing epithelium, and the concentration of IL-1β released by these PMNs.

Although PMNs release numerous cytokines, we selected IL-1β as a possible candidate for the PMN-induced recovery response because our preliminary experiments indicated that the response to PMNs was COX-dependent, and because there are studies showing that IL-1β is capable of upregulating COX-2 in gastrointestinal tissue. For example, IL-1β upregulated COX-2 in a human colonic subepithelial myofibroblast cell line (32) and
in gastric cancer cells (14). IL-1β has also been shown to increase PGE$_2$ levels in Mode-K intestinal epithelial cells (22), and IL-1β is present in gastric ulcers (39). Such IL-1β accumulation at gastric ulcers could be related to increased expression of COX-2 at the margins of ulcers, which appears to facilitate ulcer healing (33). The link between IL-1β and COX-2 is not restricted to gastrointestinal tissues. For example, IL-1β has also been shown to elevate COX-2 protein levels and PGE$_2$ production in endometrial stromal cells (40). Interestingly, IL-1 also has the capability of activating chloride secretion in T84 cells (21), and increased CFTR expression (9) which may be relevant to intestinal epithelial repair, since we have previously shown that chloride secretion is a critical signaling component of the recovery process in ischemia-injured porcine ileum (3, 28).

The remarkably rapid detection of COX-2 protein in IL-1β-stimulated mucosa could be explained by mRNA stabilization and subsequent translation (12,19). In the present study, mucosa was subjected to 45 minutes of ischemia in vivo with an additional 45 minutes of recovery time within the Ussing chambers prior to addition of IL-1β. Thus, the tissue was likely producing the COX-2 transcript prior to addition of IL-1β or PMNs. For stimuli such as IL-1β, message stabilization appears to be the primary mechanism of COX-2 induction (2, 23). Our data supports the role of IL-1β on COX-2 message stabilization as increased mRNA was detected at 5 min after 10 ng/mL IL-1β treatment. The decline in COX-2 mRNA at 15 min following IL-1β application corresponded to the elevated COX-2 protein we detected through Western blot analyses, suggesting utilization and degradation of COX-2 transcripts by this time. As far as the signaling mechanism involved in mRNA stabilization, studies in basic FGF-stimulated human intestinal epithelial cells and IL-1-stimulated intestinal myofibroblasts have suggested that p38 MAPK regulates COX-2 expression at the post-
transcriptional level by increasing mRNA stability (32). Thus, it is possible that IL-1β is capable of increasing COX-2 protein expression via a p38 MAPK-dependent mechanism resulting in mRNA stability.

The beneficial effects of IL-1β are dose specific with low doses (1ng/ml) or high doses (100ng/ml) having no effect on TER, whereas 10ng/ml consistently augmented recovery of TER. Not all studies show beneficial effects of IL-1β on intestinal tissues. For example, blockade of IL-1 and TNF-α during ischemia-reperfusion of the rat small intestine alleviated intestinal injury (43). Furthermore, addition of IL-1β directly to intestinal epithelium resulted in declining measurements of TER (6) and increases in horseradish peroxidase fluxes, which indicates altered epithelial permeability (29). Dose specificity in the present study may relate to dose-dependent effects on COX-2 expression counterbalanced by damaging effects on epithelium at higher doses. However, we did not note any change in the histologic appearance of tissues exposed to higher doses of IL-1β. A similar dose response to IL-1β has also been observed on CFTR expression in T84 cells (9). In studies by Hinterleitner et al., 10-1000pg/ml of IL-1α applied to 18Co cells dose-dependently increased PGE₂ levels with a maximum effect achieved at 100pg/ml of IL-1α, suggesting peak PGE₂ release may be achieved with sub-maximal dosages, which may explain the results in the present study. The beneficial effect of 10ng/ml IL-1β was blocked by application of a COX-2 specific inhibitor, NS-398. Additionally, at the 10ng/ml dose there was upregulation of COX-2 as determined by Western blot analysis. Furthermore, the beneficial effects of IL-1β only occurred when it was added to the serosal side of tissues, suggesting upregulation of COX-2 within cells localized to the subepithelial layer. One potential population of subepithelial cells are myofibroblasts, which have previously been shown to be capable of
expressing COX-2 when exposed to IL-1β in cell culture conditions (20, 32). Our previous studies on porcine ischemia-injured mucosa have shown expression of COX-2 in repairing epithelium, and within mononuclear cells adjacent to the epithelium (5). Based on the requirement for addition of IL-1β to the serosal surface of tissues, sub-epithelial mononuclear cells would appear to be one potential target of PMNs and IL-1β in the present studies.

The implications of this study are that PMNs have a beneficial role in recovery of ischemia-injured ileum, which must be balanced with studies indicating a deleterious role of PMNs in mucosal recovery (17). Thus, although inhibiting PMN infiltration can augment PMN recovery, other important PMN functions, such as innate defense, and upregulation of pro-reparative COX-2, suggest that this non-specific approach to blocking PMN function may have detrimental effects. Ultimately, by understanding the signaling pathways utilized by PMNs and recovering intestinal mucosa, targeted therapy that retains the beneficial elements of the inflammatory cascade may result in hastening of mucosal repair in patients suffering from a broad range of inflammatory bowel diseases.
References


15. Faour WH, He Y, He QW, Ladurantaye M, Quintero M, Mancini A, and Battista JA. Prostaglandin E2 regulates the level and stability of cyclooxygenase-2 mRNA through


Figure 1: Electrical response of ischemia-injured porcine ileal mucosa to serosal addition of neutrophils (PMNs). Tissue treated with PMNs ($5 \times 10^6$) exhibited a significant ($p<0.05$) increase in TER over time when compared to untreated ischemia-injured tissue using 2-way ANOVA on repeated measures. Pre-treatment of tissue with the COX inhibitor indomethacin (5µM) significantly ($p<0.05$) prevented the enhanced TER observed with $5 \times 10^6$ PMNs. Values represent means ± SE; n=6

Figure 2: Electrical response of ischemia-injured porcine ileal mucosa to serosal addition of PMNs, and pre-treatment with NS-398, or IL-1β receptor antagonist. Tissue pre-treated with the selective COX-2 inhibitor, NS-398 (5µM) or an IL-1β receptor antagonist (0.1mg/ml), each significantly ($p<0.05$) prevented the effect of application of $5 \times 10^6$ PMNs on TER over the experimental time course. PMN’s were added at 45 minutes. Values represent means ± SE; n=6

Figure 3: Analysis of tissue bathing solutions and stock PMN solution for IL-1β. The amount of IL-1β was significantly higher in tissue treated with PMN at 180 min compared to untreated tissue at 180 min ($^\#P<0.05$). Additionally, all tissue at 180 min had significantly higher levels of IL-1β compared to baseline (*$P<0.05$). Values represent means ± SE; n=4.

Figure 4: Dose response of ischemia-injured porcine ileal mucosa to IL-1β. Application of 10ng/ml of IL-1β significantly ($p<0.05$) enhanced TER when compared to untreated ischemia-injured mucosa over the experimental time course. IL-1β at 1ng/ml or 100ng/ml
had no effect on TER when compared to untreated ischemia-injured mucosa. Values represent means ± SE; n=7

Figure 5: Electrical response of ischemia-injured porcine ileal mucosa to treatment with IL-1β (10ng/ml) and NS-398 (5µM). Pre-treatment of tissue with the selective COX-2 inhibitor, NS-398 (5µM) significantly (p<0.05) inhibited the rise in TER following application of 10ng/ml IL-1β over the experimental time course. Values represent means ± SE; n=7

Figure 6: The effect of IL-1β on COX-2 protein expression in ischemia injured porcine ileal mucosa. COX-2 protein was assessed by Western blot analysis. Ischemia-injured ileal mucosa was treated with IL-1β (10ng/ml) or untreated. COX-2 protein expression was markedly upregulated in IL-1β-treated tissue compared to untreated ischemia-injured tissue. Shown are blots of tissue homogenates from 3 separate pigs on the same gel, representative of n=6.

Figure 7: Fold change in Cox-2 mRNA gene expression in ischemic-injured tissues subjected to IL-1β treatment. mRNA gene expression of actin and Cox-2 were measured by real time RT-PCR in ischemic-injured tissues from 2 animals (Pig 1 and 2). Tissues were either untreated or treated with IL-1β (10ng/ml) for 5, 10, or 15-min. Values represent mean (± SEM) for triplicate analyses of Cox-2 mRNA expression relative to untreated ischemic-injured tissues, adjusted for actin.
Figure 8: Transepithelial electrical resistance (TER) response of ischemia-injured porcine ileal mucosa with addition of extravasated PMNs. Tissue treated with extravasated neutrophils \(5 \times 10^6\) exhibited a significant \((p < 0.05)\) increase in TER when compared to untreated ischemia-injured tissue over the experimental time course. Values represent means \(\pm\) SE; \(n=8\)

Figure 9: The effect of PMN’s on COX-2 protein expression in ischemia injured porcine ileal mucosa. COX-2 protein was assessed by Western blot analysis. Ischemia-injured ileal mucosa was left untreated, treated with PMN \(5 \times 10^6\), IL-1\(\beta\) receptor antagonist (IL-1\(\beta\)RA), or a combination of PMN and IL-1\(\beta\)RA. COX-2 protein expression was markedly upregulated in PMN-treated tissue compared to untreated ischemia-injured tissue. Additionally, application of an IL-1\(\beta\)RA prevented the PMN-induced upregulation in COX-2 protein expression.
Shifflett et al., Figure 1

TER (Ohms.cm$^2$) vs Time (min)

Control
Isch
Isch+PMN (1x10$^6$)
Isch+PMN (5x10$^6$)
Isch+indo (5µM)
Isch+Indo+PMN (5x10$^6$)

PMN Added

0 30 60 90 120 150 180

0 20 40 60 80
Figure 2

- Ischemic
- Ischemic+PMN (5x10^6)
- Isch+NS398 (5µM)
- Ischemic+PMN+NS398
- Ischemic+PMN+IL1βra (0.1µg/ml)

PMN added
Shifflett et al., Figure 5

![Graph showing TER (Ohms/cm²) over time for different conditions: Ischemia, Ischemia + NS398 (5µM), Ischemia + NS398 + IL-1β (10ng/mL), and Ischemia + IL-1β (10ng/mL). The x-axis represents time in minutes (0-180), and the y-axis represents TER in Ohms/cm². An arrow indicates the time when IL-1β is added.]

- **Ischemia**
- **Ischemia + NS398 (5µM)**
- **Ischemia + NS398 + IL-1β (10ng/mL)**
- **Ischemia + IL-1β (10ng/mL)**
Shifflett et al., Figure 6
<table>
<thead>
<tr>
<th>Time Following IL-1β Addition (minutes)</th>
<th>COX-2 mRNA Expression (Fold Change)</th>
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<tbody>
<tr>
<td>5</td>
<td>Pig 1: 15</td>
</tr>
<tr>
<td>10</td>
<td>Pig 1: 20, Pig 2: 1</td>
</tr>
<tr>
<td>15</td>
<td>Pig 1: 0, Pig 2: 0.5</td>
</tr>
</tbody>
</table>

Shifflett et al. Figure 7
Shifflett et al., Figure 9

1. Ischemic
2. Ischemic+PMN
3. Ischemic+IL-1βRA
4. Ischemic+PMN+IL-1βRA
CHAPTER FOUR

MITOGEN ACTIVATED PROTEIN KINASES REGULATE COX-2 AND MUCOSAL RECOVERY IN ISCHEMIC-INJURED PORCINE ILEUM

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Short Title: MAPK’s regulate recovery of ischemic-injured ileum

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Abstract

Shifflett, Donnie E., Samuel L. Jones, Adam J. Moeser, and Anthony T. Blikslager. Mitogen activated protein kinases regulate COX-2 and mucosal recovery in ischemic-injured porcine ileum - Mitogen activated protein kinase (MAPK) pathways transduce signals from a diverse array of extracellular stimuli. The three primary MAPK signaling pathways are the extracellular regulated kinases (ERK 1&2), p38 MAPK, and eJun NH₂-terminal kinase (JNK). Previous research in our laboratory has shown that COX-2-elaborated prostanoids participate in recovery of mucosal barrier function in ischemic-injured porcine ileum. Because COX-2 expression is regulated in part by MAPK’s, we postulated that MAPK pathways would play an integral role in recovery of injured mucosa. Porcine mucosa was subjected to 45-minutes of ischemia, after which tissues were mounted in Ussing chambers, and transepithelial electrical resistance (TER) was monitored as an index of recovery of barrier function. Treatment of tissues with the p38 MAPK inhibitor SB-203580 (0.1mM), or the ERK 1&2 inhibitor, PD-98059 (0.1mM), abolished recovery. Western blot analysis revealed that SB-203580 inhibited upregulation of COX-2 that was observed in untreated ischemic-injured mucosa, whereas PD-98059 had no effect on COX-2 expression. Inhibition of TER recovery by SB-203580 or PD-98059 was overcome by administration of exogenous PGE₂ (1µM). The JNK inhibitor, SP-600125 (0.1mM), significantly increased TER and resulted in COX-2 upregulation. COX-2 expression appears to be positively and negatively regulated by the p38 MAPK and the JNK pathways respectively. Alternatively, ERK 1&2 appear to be involved in COX-2-independent reparative events that remain to be defined.

Key Words: intestine, prostaglandin, transepithelial electrical resistance, mitogen-activated protein kinase
Introduction

The mitogen-activated protein kinases (MAPK’s) are an evolutionarily conserved family of enzymes that form a highly integrated network to regulate cellular functions such as differentiation, proliferation and cell death. Thus, MAPK’s transduce extracellular signals into intracellular responses. At least three distinct MAPK groups have been identified: extracellular regulated kinases 1&2 (ERK 1&2), p38 MAPK, and c-Jun-NH$_2$ terminal kinase (JNK). MAPK signaling cascades are viewed as parallel pathways, although cross-talk may exist.(18,34) Upon phosphorylation and subsequent activation of a MAPK, the MAPK can enter the cell nucleus and activate transcription factors. This ultimately results in expression of target genes and a biological response.(15)

One biologically responsive gene is $\text{cox-2}$. (15,26,33) There are three known isoforms of cyclooxygenase (COX): COX-1, COX-2, and COX-3. (10,28) While COX-1 is constitutively expressed in most tissues, COX-2 is inducible under a variety of conditions, including exposure to select cytokines and growth factors, (16,27) experimental colitis, (1) and ischemia. (8) Both COX-1 and COX-2 appear to play a role in recovery of ischemic-injured intestinal mucosa (8,23) and in colonic mucosal defense. (25,30) Furthermore, COX-2 is expressed in the epithelium at the margins of experimentally-induced gastric ulcers in rats, and selective inhibition of COX-2 retards epithelial repair. (23) Alternatively, while inhibition of COX-2 did not interrupt recovery of barrier function in ischemic-injured mucosa in previous studies from our laboratory, COX-2 prostanoids were able to stimulate recovery in the absence of COX-1 prostanoids. (8) In additional studies from our laboratory, we noted that IL-1$\beta$ was able to enhance recovery of ischemic-injured ileal mucosal in a COX-2-dependent fashion.
Previous research examining the role of the MAPK pathways in regulating COX-2 has yielded conflicting results. In various intestinal epithelial cell lines, including I407, Caco-2, and HT-29 cells, that basic fibroblast growth factor (bFGF) increases COX-2 expression via p38 MAP kinase.(32) However, in porcine aortic smooth muscle cells, bFGF-induced COX-2 expression was inhibited by PD-98059, an ERK pathway inhibitor, indicating an important role for ERK 1&2.(19) In rat mesangial cells, JNK and p38 MAPK mediate IL-1β-stimulated COX-2 expression(13,14) while ERK and p38 MAPK regulate COX-2 expression induced by TGF-α and IFN-γ in human epidermal keratinocytes and squamous carcinoma cells.(21) In NIH3T3 cells, regulation of COX-2 by platelet-derived growth factor is mediated through the ERK and JNK pathways.(35) Thus, it is apparent that the MAPK pathways are critical in regulating COX-2 expression, but there is considerable variability in this regulation dependent upon the cell type and stimulus.

The purpose of the present study was to investigate the role of the MAPK pathways in regulating recovery of porcine ischemic-injured mucosa. We postulated that MAPK pathways are intimately involved in mucosal recovery of acutely injured porcine ileum via regulation of COX-2 expression.
Materials and Methods

Experimental surgery

All procedures were approved by the North Carolina State University Institutional Animal Care and Use Committee, and have been previously described in detail.(5,6,7) Briefly, 6-8 week-old Yorkshire-cross pigs of either sex were anesthetized using a combination of xylazine (1.5mg/kg) and ketamine (11mg/kg). Pigs were intubated via a tracheostomy, placed on a heating pad and ventilated with 100% O₂ via a tracheotomy using a time-cycled ventilator. Anesthesia was maintained using periodic IV administration of a 5% sodium thiopental solution via a jugular catheter. Maintenance fluids were administered IV at a rate of 15ml/kg/hr throughout the surgery. The ileum was located via a midline incision, after which 10cm ileal segments were ligated and subjected to ischemia by ligating the local mesenteric blood supply. Additional 10-cm ileal loops not subjected to ischemia were utilized as control tissues. After 45-minutes ischemia, pigs were euthanized with an overdose of pentobarbital and 10cm ileal loops were promptly removed and placed in oxygenated Ringers solution (95% O₂/5% CO₂).

Ussing chamber studies

The mucosa was stripped from the seromuscular layer and mounted in 3.14 cm² aperture Ussing chambers. The tissues were bathed on both serosal and mucosal sides with 10ml of oxygenated (95% O₂/5% CO₂) Ringer’s solution. In addition, the serosal bathing solution contained 10mM of glucose, which was osmotically balanced by 10mM mannitol on the mucosal side. Bathing solutions were circulated in water-jacketed reservoirs, and maintained at 37°C. The spontaneous potential difference (PD) was measured using Ringer-
agar bridges connected to calomel electrodes, and the PD was short-circuited through Ag-AgCl electrodes using a voltage clamp that corrected for fluid resistance. Resistance (Ω·cm²) was calculated from the spontaneous PD and short-circuit current (Isc). If the spontaneous PD was between -1.0 and 1.0mV, tissues were current clamped at ±100µA for 5 seconds and the PD recorded. Short-circuit current and PD were recorded every 15-minutes for 180-minutes.

**Experimental treatments**

The JNK inhibitor, SP-600125 was purchased from BioMol (Plymouth Meeting, PA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

**Histology**

Tissues were taken immediately following ischemia and following the 180-minute recovery period for histological evaluation. Tissues were sectioned (5µm) and stained with hematoxylin and eosin. For each tissue, an investigator blinded to the treatment group evaluated 3 sections. The height of the villus, and the width at the midpoint of the villus were obtained using a light microscope with an ocular micrometer. For height measurements, the base of the villus was defined as the intersection between adjacent villi at the opening of the crypt. For villi in which the height of one side of the villus was disparate from the other side the average height was recorded. In addition, the height of the epithelial-covered portion of each villus was measured. The surface area of the villus was calculated using the formula for the surface area of a cylinder. The formula was modified by subtracting the area of the base of the villus, and multiplying by a factor accounting for the variable position at which each
villus was cross-sectioned. In addition, the formula was modified by a factor that accounted for the hemispherical shape of the upper portion of the villus. The percentage of the villous surface area that remained denuded was calculated from the total surface area of the villus and the surface area of the villus covered by epithelium. The percent-denuded villus surface area was used as an index of epithelial restitution.

**Gel electrophoresis and Western blotting**

Tissues for total MAPK and phospho-MAPK Western blots were taken immediately after ischemia and prior to mounting in the Ussing chambers. Tissues mounted in the Ussing chambers were removed upon completion of the collection of electrical data (180-minutes) for COX-2 Western blots. Mucosal scraping was performed in order to remove additional connective tissue, after which tissue was snap-frozen in liquid nitrogen and stored at –70°C until analysis for COX-2 protein. In preparation for SDS-PAGE, tissues were thawed to 4°C. Tissue portions were added to 1ml of chilled RIPA buffer (0.15M NaCL, 50 mM Tris [pH 7.2], 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS), including protease inhibitors (PMSF and aprotinin). The mixture was homogenized on ice and then centrifuged twice at 10,000g for 10-minutes at 4°C and the supernatant saved. Protein analysis of extract aliquots was performed (DC protein assay, Bio-Rad, Hercules, CA). Tissue extracts (amounts equalized by protein concentration) were mixed with an equal volume of 2x SDS-PAGE sample buffer and boiled for 4-minutes at 100°C. Lysates were loaded on a 10% SDS-polyacrylamide gel and electrophoresis was carried out according to standard protocols. Proteins were transferred to a nitrocellulose membrane (Hybond ECL, Amersham Life Science, Birmingham, UK) using an electroblotting mini-transfer apparatus according to the
manufacturer’s protocol. Membranes were blocked in Tris buffered saline (TBS) containing 0.05% Tween (TBS-T) and 5% dry powered milk. Membranes were washed three times for 5-minutes each with (TBS-T) and incubated for 2 hr at room temperature in primary antibody derived from a rabbit with porcine reactivity. After washing three times for 5-minutes each with TBS-T, the membranes were incubated for one hour with horseradish peroxidase conjugated secondary antibody. After washing two times for 5-minutes each with TBS-T and one time with TBS for 15-minutes, the membranes were developed for visualization of protein by addition of enhanced chemiluminescence reagent (Amersham, Princeton NJ).

**Data analysis**

All data were analyzed using a statistical software package (Sigmastat, Jandel Scientific, San Rafael, CA). Data were reported as mean ± standard error of the mean (SE) for a given number (‘n’) of animals for each experiment. Results were analyzed by 2-way ANOVA on repeated measures. For analyses where significance was detected by ANOVA, the Tukey’s test was utilized for post-hoc pairwise multiple comparisons. The α level for statistical significance was set at p<0.05. Where a significant time x treatment interaction was noted, a one-way ANOVA was utilized to differentiate individual treatment effects.
Results

Effect of ischemia on activation of MAPK’s

In order to clarify the role of the MAPK’s in regulating recovery from ischemia, we first sought to determine if subjecting ileal mucosa to ischemic injury resulted in MAPK activation. Accordingly, mucosa scraped from normal ileum (control) and ileum exposed to 45-minutes ischemia was Western blotted for the total levels of each of the three primary MAPK’s and the phosphorylated (activated) forms of MAPK’s (Fig. 1). Total protein levels for each of the MAPK’s were unchanged in ischemic-injured tissue, whereas ischemia resulted in marked increases in the phosphorylated forms of p38 MAPK, ERK 1&2, and JNK when compared to control mucosa (Fig. 1).

In vitro Recovery of Ischemic-Injured Ileal Mucosa

Porcine ileum was subjected to 45-minutes of ischemia in vivo, after which normal (control) and ischemic-injured mucosa was removed and monitored over 180-minutes in Ussing chambers. Histological analyses revealed loss of approximately 20% of the apical villous epithelium following ischemia, and restitution was complete within 60-minutes (data not shown), as reported in previous publications from our laboratory.(5,6) Ischemic-injured mucosa had TER values that were significantly lower than control values and mucosal-to-serosal fluxes of mannitol that were significantly higher than control tissues during the early part of the recovery period (Fig. 2). Ischemic-injured tissues recovered control levels of these indices of barrier function within 120-135-minutes. The latter phases of recovery were
presumed to result from recovery of paracellular resistance because of the early completion of restitution, as shown in previous studies.(5,6)

The Effect of ERK 1&2 Inhibition on TER

In further studies, we sought to determine the role of MAPK’s in recovery of TER in vitro. A range of doses (0.1mM –1µM) of the ERK 1&2 inhibitor, PD-98059, was applied to ischemic-injured tissues, resulting in dose-specific inhibition of recovery of TER (Fig 3A). PD-98059 significantly (p<0.05) inhibited recovery at a dose of 0.1mM whereas lower doses (0.01mM and 1µM) had no effect on recovery of ischemic-injured mucosa. Uninjured tissue was also exposed to 0.1mM PD-98059, which had no effect (data not shown). Histological analysis revealed that ERK 1&2 inhibition had no effect on histologic indices of restitution when compared to untreated ischemic-injured mucosa.

Because we have previously shown that recovery of TER in this tissue is largely regulated by prostaglandins (PGs),(5,6,7) we next wanted to determine if we could reverse the effect of PD-98059 by addition of exogenous 16,16-dimethyl prostaglandin E₂ (PGE₂). PGE₂ at a dose of 1µM caused a significant (p<0.05) increase in TER of ischemic-injured ileum when added alone, and PGE₂ also overcame inhibition of TER by 0.1mM PD-98059 (Fig. 3B). However, tissues treated with PGE₂ and PD-98059 did not reach the same levels as tissues treated with PGE₂ alone, suggesting a continued partial inhibitory effect of PD-98059 on mucosal recovery.

In previous experiments on porcine ileal mucosa, we have shown upregulation of COX-2 following ischemic injury, and we have shown that COX-2 participates in recovery of TER.(7) Therefore, we wanted to examine the possibility that ERK inhibition decreased
COX-2 expression. However, there was no effect of PD-98059 on COX-2 protein expression levels (Fig. 3C). Thus, blockade of TER recovery with ERK 1&2 inhibition appeared to be independent of COX-2 expression.

**The Effect of p38 MAPK Inhibition on TER**

In order to determine if other signaling kinases played a role in recovery of ischemic-injured mucosa, the p38 MAPK inhibitor, SB-203580 was placed on ischemic-injured porcine ileum. Mucosal recovery was inhibited in a dose-dependent fashion with SB-203580 (Fig. 4A). Similar to ERK 1&2 inhibition, less concentrated doses of the p38 MAPK inhibitor (0.01mM and 1µM) had no effect on TER when compared to untreated ischemic-injured porcine ileum, whereas 0.1mM SB-203580 significantly inhibited recovery of TER. Uninjured tissue was also exposed to 0.1mM SB-203580, but there were no observable effects (data not shown). Additionally, histological analysis revealed that p38 inhibition had no significant effect on restitution when compared to untreated ischemic-injured mucosa.

Addition of 1µM PGE₂ to ischemic-injured tissue significantly (p<0.05) reversed the inhibitory effect of 0.1mM SB-230580 (Fig. 4B). Western blot analysis was also performed for COX-2 protein expression in untreated ischemic-injured ileum and in tissue treated with SB-203580. In contrast to our experiments with the ERK inhibitor PD-98059, 0.1mM SB-203580 decreased COX-2 protein expression compared to untreated ischemic-injured tissue (Fig. 4C).

**The Effect of JNK Inhibition on TER**
Since we had noted inhibition of COX-2 expression and recovery of TER with an inhibitor of p38 MAPK, we also wanted to assess the other stress-activated protein kinase pathway, c-Jun NH$_2$ terminal kinase (JNK). Low doses (0.01mM and 1µM) of the JNK inhibitor SP-600125 had no effect on recovering ischemic-injured ileum, similar to the responses we observed with low doses of ERK 1&2 and p38 inhibitors. Conversely, we observed a significant (p<0.05) increase in recovery of TER over time with a more concentrated dose (0.1mM) of SP-600125 (Fig. 5A). SP-600125 (0.1mM) was also placed on uninjured tissue and had no effects (data not shown). Additionally, histological analysis revealed that JNK inhibition had no significant effect on restitution when compared to untreated ischemic-injured mucosa.

PGE$_2$ (1µM) was utilized in combination with SP-600125 to determine if there would be an additive increase in TER. The combination of 0.1mM SP-600125 and 1µM PGE$_2$ caused a significant (p<0.05) increase in TER compared to either treatment alone (Fig. 5B). Western blot analysis for COX-2 protein expression in untreated ischemic-injured porcine ileum and in ischemic-injured ileum treated with 0.1mM SP-600125 showed that the increase in TER observed with 0.1mM SP-600125 correlated with increased expression of COX-2 (Fig. 5C). Furthermore, the increase in TER observed with 0.1mM SP-600125 was blocked by addition of a COX-2 selective inhibitor, NS-398 (5µM) (Fig. 6).

**The Effect of Dual Inhibition of JNK and p38 MAPK on TER**

Our data showed that JNK inhibition increases TER, while p38 MAPK inhibition resulted in blockade of TER when compared to untreated ischemic-injured mucosa. Thus, we next sought to determine if one of these pathways had a more dominant role in recovering
ischemic-injured ileum. Accordingly, p38 MAPK inhibition with SB-203580 (0.1mM) abolished recovery of TER, application of the JNK inhibitor SP-600125 (0.1mM) significantly increased peak TER, whereas a combination of SP-600125 and SB-203580 resulted in an inhibitory response similar to SB-203580 treatment alone (Fig. 7A). Furthermore, dual inhibition of p38 MAPK and JNK resulted in reduced COX-2 protein expression (Fig. 7B).

Discussion

MAPK’s form a highly integrated network to regulate various cellular processes. Using a porcine model of intestinal ischemia we have shown that: 1) ERK 1&2 and p38 MAPK positively regulate recovery from ischemia while JNK negatively regulates recovery from ischemia; 2) ERK 1&2 do not regulate COX-2 expression while p38 MAPK and JNK positively and negatively regulate COX-2 protein expression, respectively; and 3) p38 MAPK appears to have a dominant role over JNK in regulating recovery from ischemia. There has been recent intensive clinical interest in inhibition of p38 MAPK for a variety of clinical conditions. For example, there have been more than 48 patent applications from more than 15 pharmaceutical companies for inhibitors of p38 MAPK. Inhibitors of p38 MAPK are being developed for various conditions, including rheumatoid arthritis, psoriasis, and inflammatory lung diseases. (9) Furthermore, p38 MAPK and JNK inhibition have been evaluated for treatment of Crohn’s disease. CNI-1493, an inhibitor of both p38 MAPK and JNK, resulted in endoscopic healing and significant clinical improvement in patients suffering from Crohn’s disease. (17) Despite the fact that CNI-1493 purportedly inhibits p38 MAPK and JNK, the in vivo effects of CNI-1493 appeared to be attributable to a reduction in JNK activity. This would lend support to our study, which showed that inhibition of JNK
results in enhanced recovery barrier function, whereas inhibition of p38 MAPK had a deleterious effect on mucosal recovery from ischemia.

Mechanisms of mucosal recovery in our model of porcine ischemic injury appear to relate predominantly to recovery of paracellular resistance. For instance, there was no effect of any of the treatments in the present study on epithelial restitution, despite significantly differing effects on TER. Furthermore, restitution is complete by 60-minutes, prior to full recovery of TER. In addition, we have previously shown morphologic evidence of tight junction closure in response to PG administration,(5,6) and recovery of TER is correlated with reductions of mucosal-to-serosal flux of the paracellular probe mannitol. The nature of mucosal recovery in vitro, as studied in the present series of experiments, may differ substantially from recovery noted in vivo. For example, in vitro recovery is a rapid process completed within 180-minutes, whereas in vivo recovery is not as rapid, requiring 4-6-hours to complete, most likely because of the in vivo effects of neutrophils.(12) Thus, the in vitro model abolishes any effects of the classic pathway of reperfusion injury, which involves infiltration of neutrophils. However, it must be acknowledged that in vitro recovery does constitute re-oxygenation of tissues using solutions gassed with a 95%O₂/5% CO₂ mixture. This re-oxygenation may provide the beneficial effects of reperfusion (return of an O₂ source) without the deleterious effects of mucosal inflammation.

The present study suggests a role for COX-2 in mucosal recovery from ischemic injury. Previous studies have indicated that COX-2 is important for mucosal recovery in porcine ischemic-injured intestinal mucosa(8) and acetic acid-induced gastric ulcers in rodents.(23) Evidence in support of the importance of COX-elaborated prostanoids in mucosal recovery from ischemic injury from our previous studies includes inhibition of
recovery by treatment with the non-selective COX inhibitor indomethacin or treatment with the COX-1 and COX-2 inhibitors SC-560 and NS-398 respectively, and restoration of recovery following these treatments with addition of PGE\textsubscript{2}. More specifically, PGE\textsubscript{2} appears to act via cAMP, a second messenger that has previously been shown to augment paracellular resistance.\textsuperscript{11} For example, administration of PGE\textsubscript{2} resulted in an approximately two-fold increase in tissue cAMP levels,\textsuperscript{5} and selective PGE receptor agonists and antagonists suggested a role for G\textsubscript{s}-linked prostanoid receptors.\textsuperscript{4} Furthermore, addition of the cAMP agonist, VIP, or 8-bromo-cAMP simulated the PGE\textsubscript{2}-induced recovery response.\textsuperscript{6} However, not all of the effects of the MAPK’s shown in the present study necessarily have a direct link to prostanoid effects on mucosal recovery, despite demonstrated effects on COX-2 expression. For example, PGE\textsubscript{2} administration restored baseline levels of mucosal recovery in the presence of p38 MAPK inhibition, but did not raise TER to levels in the presence of PGE\textsubscript{2} alone by the end of the recovery period. Thus, PGE\textsubscript{2} and p38 MAPK may be operating in parallel rather than sequential pathways. The evidence in support of a direct role of JNK in prostanoid pathways is stronger, since inhibition of JNK enhanced COX-2 expression and recovery of TER, and JNK-enhanced mucosal recovery was inhibited by a selective COX-2 inhibitor. On the other hand, ERK pathways appear to regulate mucosal recovery without affecting COX-2 expression.

Regardless of the exact role of COX-2 in mucosal recovery, the present data provides insight into upstream regulators of COX-2 expression. The p38 MAPK regulates COX-2 expression at the post-transcriptional level by increasing mRNA stability in basic FGF-stimulated human intestinal epithelial cells \textsuperscript{32} and IL-1-stimulated intestinal myofibroblasts.\textsuperscript{22} In contrast, Guo et al have shown that bombesin-induced p38 MAPK
activation regulates COX-2 transcription rather than mRNA stability.(15) In agreement with our work, Tessner et al reported that p38 MAPK positively regulates COX-2 protein expression.(32) In our model of intestinal ischemia, we have shown that p38 MAPK inhibition blocks upregulation of COX-2 protein expression, which appears to be critical for recovery.

Recent work also suggests that ERK 1&2 can regulate COX-2 expression. For example, ERK 1&2 inhibition suppresses COX-2 protein expression by approximately 76% in a human colonic subepithelial myofibroblast cell line stimulated with IL-1(22) and inhibits bombesin and gastrin-stimulated COX-2 mRNA levels in rat intestinal epithelial cells.(15) In contrast, ERK 1&2 inhibition has no effect on COX-2 protein expression in Caco-2 cells,(2) and previous reports have demonstrated no effect of ERK 1&2 inhibition on COX-2 mRNA stability.(22,24) Data presented herein demonstrates that ERK 1&2 inhibition has no effect on COX-2 protein expression in ischemic-injured porcine ileal mucosa, yet is capable of suppressing recovery. Previous research from our laboratory has shown that recovery of TER is largely correlated with recovery of paracellular resistance, and prostanoids have no perceptible effect on epithelial restitution. Similarly in the present report, ERK inhibition had no effect on early reparative events of villous contraction and restitution, while markedly inhibiting recovery of TER. Thus, it can be speculated that ERK 1&2 is regulating some component of tight junction re-assembly or closure. As an example of this potential mechanism, inhibition of ERK 1&2 inhibits claudin-2 mRNA and protein expression in T84 cells exposed to IL-17.(20) It should be noted that in the present study, PGE2 was able to partially reverse the inhibitory effects of the ERK inhibitor PD-98059. However, tissues treated with PGE2 and PD-98059 failed to reach levels of TER seen in tissues exposed to
PGE$_2$ alone, suggesting a continued PG effect independent of the effect of the ERK inhibitor on mucosal recovery.

Studies examining the regulation of COX-2 via the JNK pathway are limited. Moon and Pestka have shown that impairment of JNK function through a dominant negative JNK vector has no effect on COX-2 protein expression.(24) It has also been shown that JNK has no effect on COX-2 mRNA stability in cells stimulated with endogenous promoters of gastrointestinal cancer.(36) We report here that pharmacological inhibition of JNK increases COX-2 protein expression in ischemic-injured ileal mucosa. From this finding, it can be speculated that JNK has a down regulatory effect on COX-2 protein expression. The fact that the beneficial effect of SP-600125 on TER was blocked by the selective COX-2 inhibitor NS-398 further supported a role for JNK in the regulation of COX-2. In addition, the effects of SP-600125 on TER and the effect of exogenous PGE$_2$ on TER were approximately equal, whereas PGE$_2$ in combination with SP-600125 had an additive beneficial effect on TER.

Finally, we report that p38 MAPK appears to have a more dominant role in regulating recovery from intestinal ischemia than JNK. The increase in recovery of TER observed upon inhibition of JNK was abolished when the p38 MAPK inhibitor, SB-203580, was added in combination with the JNK inhibitor, SP-600125. The concept of cross communication among the three primary MAPK pathways has been previously reported. For example, in corneal epithelial cells, evidence exists that cross talk activation of p38 MAPK and ERK 1&2 occurs during corneal wound healing.(31) Inhibition of either p38 MAPK or ERK 1&2 induced cross-activation of the other. Furthermore, inhibition of JNK suppressed TGF-β activation of p38 MAPK in FaO rat hepatoma cells.(29) Based upon the data presented herein, we speculate that there is cross-talk between JNK and p38 MAPK in the regulation of COX-2,
with p38 MAPK activation resulting in enhanced expression of COX-2, possibly by inhibiting the JNK pathway.
References


Figure 1: Western blot analysis of intestinal mucosa subjected to 45-minutes ischemia to assess total levels of each of the three primary MAPK’s and their phosphorylation status. Ischemia did not appear to change total protein levels of each of the three primary MAPK’s, whereas ischemia resulted in marked increases in phosphorylation of p38 MAPK, ERK1&2 (p42 and p44) and JNK (p54 and p46). Blot is representative of n=4 blots on separate animals.

Figure 2: Recovery of barrier function of ischemic-injured porcine ileal mucosa as assessed by changes in TER and mucosal-to-serosal fluxes of mannitol. A. At the beginning of the recovery period, the TER of ischemic-injured mucosa was significantly below that of uninjured control tissues (*p<0.05). Ischemic-injured mucosa reached TER values not significantly different from control mucosa by 135-minutes. B. Corresponding mucosal-to-serosal fluxes of $^3$H-mannitol revealed significant increases in flux of this paracellular probe by 60-minutes of the recovery period, whereas fluxes reached levels not significantly different from control by 120-minutes (Fig. 2B, *P<0.05). Values represent means ± SE; n=8.

Figure 3: Response of ischemic-injured porcine ileal mucosa to addition of the MEK inhibitor, PD-98059. A. There was significant inhibition of recovery of TER in the presence of 0.1mM PD-98059 by the end of the recovery period (*p<0.05), whereas less concentrated doses of PD-98059 had no effect on TER when compared to untreated ischemic-injured mucosa. Values represent means ± SE; n=8. B. Recovery of TER in tissue treated with 0.1mM PD-98059 was significantly suppressed (*p<0.05) when compared to untreated
ischemic-injured mucosa, whereas ischemic-injured tissue treated with PGE$_2$ had a significant increase (${^\dagger}P<0.05$) in TER. Tissue treated with 1µM PGE$_2$ in the presence of PD-98059 had a significantly ($\#P<0.05$) increased TER compared to untreated ischemic-injured tissue, but did not attain TER values of PGE$_2$ alone. Values represent means ± SE; n=8. C. Western analysis for COX-2 protein expression indicated that COX-2 was upregulated in ischemic-injured mucosa when compared to control mucosa. However, COX-2 protein expression appeared unchanged in tissue treated with 0.1mM PD-98059 when compared to untreated ischemic-injured tissue. Blot is representative of 4 experiments.

Figure 4: Response of ischemic-injured porcine ileal mucosa to addition of the p38 MAPK inhibitor, SB-203580. A. Addition of 0.1mM SB-203580 to ischemic-injured mucosa significantly suppressed recovery of TER ($*$p<0.05) while less concentrated doses of SB-203580 had no effect on TER when compared to untreated ischemic-injured mucosa. Values represent means ± SE; n=8. B. Recovery of TER in tissue treated with 0.1mM SB-203580 was significantly suppressed ($*$p<0.05) when compared to untreated ischemic-injured mucosa, whereas ischemic-injured tissue treated with PGE$_2$ had a significant increase (${^\dagger}P<0.05$) in TER. Tissue treated with 1µM PGE$_2$ in the presence of SB-203580 had a significantly ($\#P<0.05$) increased TER compared to untreated ischemic-injured tissue, but did not attain TER values of PGE$_2$ alone. Values represent means ± SE; n=8. C. Western analysis for COX-2 protein expression indicated that COX-2 protein expression was reduced in tissue treated with 0.1mM SB-203580 when compared to untreated ischemic-injured tissue. Blot is representative of 4 experiments.
Figure 5: Response of ischemic-injured porcine ileal mucosa to addition of the JNK inhibitor, SP-600125. **A.** Addition of 0.1mM SP-600125 to ischemic-injured mucosa significantly enhanced recovery of TER (\(^*p<0.05\)) while less concentrated doses of SP-600125 had no effect on TER when compared to untreated ischemic-injured mucosa. Values represent means ± SE; n=8. **B.** Recovery of TER in tissue treated with 0.1mM SP-600125 was significantly increased (\(^*p<0.05\)) when compared to untreated ischemic-injured mucosa. Similarly, addition of 1µM PGE\(_2\) significantly increased TER (\(^*P<0.05\)) to levels similar to those of tissues treated with SP-600125. Treatment with both 0.1mM SP-600125 and 1µM PGE\(_2\) resulted in significant (\(^{#}P<0.05\)) additive increases in TER. Values represent means ± SE; n=8. **C.** Western analyses indicated that COX-2 protein expression was increased in tissue treated with 0.1mM SP-600125 when compared to untreated ischemic-injured tissue. Blot is representative of 4 experiments.

Figure 6: Electrical response of ischemic-injured porcine ileal mucosa to addition of the JNK inhibitor, SP-600125, and the selective COX-2 inhibitor, NS-398. Recovery of TER was significantly increased (\(^*p<0.05\)) in tissue treated with 0.1mM SP-600125 when compared to untreated ischemic-injured mucosa, and this increase was significantly inhibited (\(^{*}p<0.05\)) by pre-treatment with the COX-2 inhibitor NS-398 (5µM). Values are means ± SE; n=8.

Figure 7: Effect of treatment of ischemic-injured porcine ileal mucosa with the p38 MAPK inhibitor SB-203580 and the JNK inhibitor SP-600125. **A.** Treatment with 0.1mM SB-203580 resulted in a significant decrease (\(^{*}P<0.05\)) in TER, whereas 0.1mM SP-600125
caused a significant increase (*P<0.05) in TER when compared to untreated ischemic-injured tissue. Alternatively, treatment with a combination of SB-203580 and SP-600125 significantly reduced (*P<0.05) peak TER when compared to untreated ischemic-injured tissue, similar to TER levels in tissues treated with SB-203580 alone. Bars represent means ± SE; n=8. **B.** Western analyses revealed reduced COX-2 expression in ischemic-injured ileal mucosa treated with 0.1 mM SB-203580 when compared to untreated tissue, whereas treatment with 0.1mM SP-600125 resulted in increased levels of COX-2 protein expression. The combination of p38 MAPK inhibition (SB-203580) and JNK inhibition (SP-600125) resulted in decreased levels of COX-2 protein expression when compared to untreated ischemic-injured mucosa. The level of COX-2 protein with dual inhibition was similar to COX-2 protein levels with p38 MAPK inhibition alone. Blot is representative of 4 experiments.
Shifflett et al. Figure 3

A

- Isch
- Isch+PD98059 (0.1mM)
- Isch+PD98059 (0.01mM)
- Isch+PD98059 (1µM)

B

- Isch
- Isch+PD98059 (0.1mM)
- Isch+PGE\(_2\) (1µM)
- Isch+PD98059 (0.1mM) + PGE\(_2\) (1µM)

TER (Ω.cm\(^2\))

Time (min)
A

B

TER (Ω.cm²)

Time (min)

Add PGE₂

Add PGE₂
Shifflett et al. Figure 4C

COX-2

CONT ISCH ISCH+SB
A

TER (Ω cm²)

Isch
Isch+SP600125 (0.1mM)
Isch+SP600125 (0.01mM)
Isch+SP600125 (1µM)

B

TER (Ω cm²)

Isch
Isch+PGE₂ (1µM)
Isch+SP600125+PGE₂

Add PGE₂

Time (min)
Shifflett et al. Figure 5C
Shifflett et al. Figure 6

The graph shows the time course of TER (Ω.cm²) measured over 180 minutes for different conditions: Isch, Isch+SP600125 (0.1mM), Isch+NS398 (5µM), and Isch+NS398+SP600125. The y-axis represents TER values ranging from 10 to 70, and the x-axis represents time in minutes from 0 to 180. The data points are accompanied by error bars indicating the variability of measurements. Significant differences are indicated by asterisks (*) for Isch+SP600125 and a plus sign (+) for Isch+NS398+SP600125.
Shifflett et al. Figure 7A

Shifflett et al. Figure 7B
CHAPTER FIVE

PGE$_2$ INDUCES INCREASES IN TRANSEPITHELIAL RESISTANCE AND A SHIFT IN CLAUDIN-1 DISTRIBUTION VIA CHLORIDE SECRETORY PATHWAYS IN HUMAN COLONIC T84 CELLS

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Abstract

Using a porcine model of ileal ischemic injury, we have previously shown that exogenous PGE$_2$ administration induces an immediate, sharp rise in short circuit current (Isc), indicative of chloride secretion, followed by a more gradual increase in transepithelial resistance (TER) that is associated with closure of tight junctions within restituted epithelium. However, the mechanism by which PGE$_2$ rescues TER is unknown. Furthermore, the contribution of PGE$_2$-elicited chloride secretion to alteration of tight junction structure has yet to be determined. In order to study these mechanisms, we utilized T84 cells, which have well characterized chloride secretory pathways and form tight junctions. We postulated that PGE$_2$ would induce a sharp increase in Isc that would precede increases in TER associated with re-distribution of TJ proteins. T84 cells were grown to confluence on Transwell membranes, but utilized at lower TER values (200-500 ohms.cm$^2$) to simulate our ileal mucosal model of “leaky” restituted epithelium. Triton-X soluble and insoluble proteins were extracted following application of treatments for analysis of the distribution of TJ integral membrane proteins. Immunoflourescence analysis was also performed to visualize TJ proteins. Basolateral application of PGE$_2$ (1µM) induced an immediate and significant sharp increase in Isc. Significant increases in TER were observed in PGE$_2$-treated cells beginning 30 min after PGE$_2$ administration, and were sustained for up to 240-minutes after PGE$_2$ administration. Although PGE$_2$ induced similar Isc changes in high resistance cells (>1200 ohms.cm$^2$), there was a corresponding decrease in TER values. In further studies on leaky epithelial cells, basolateral application of the Na$^+$/K$^+$/2Cl$^-$ cotransporter inhibitor bumetanide (0.1mM), abolished the PGE$_2$-induced rise in Isc and the subsequent elevations in TER. PGE$_2$ induced a shift in claudin-1 from the Triton-X soluble to insoluble fraction, beginning 4-hour after
PGE$_2$ administration and this effect was prevented by pre-treatment with bumetanide. Furthermore, there were no changes in the distribution of occludin or claudins-3 and -5 in response to any of the treatments. These data were further supported via immunofluorescence. Additionally, we showed that PGE$_2$ induced tyrosine phosphorylation of claudin-1 by 30-minutes, an effect prevented by bumetanide. These data indicate that PGE$_2$-induced chloride secretion in T84 cells is directly linked with increases in TER, and these elevations in TER are associated with phosphorylation of claudin-1, a shift in claudin-1 to the tight junction region via immunofluorescence analysis and a shift in Triton-X solubility. These studies suggest that PGE$_2$ utilizes chloride secretory pathways as a signal transduction system to alter tight junction structure in order to increase epithelial barrier function in leaky epithelia.
Introduction

The intestinal epithelium provides a barrier to luminal solutes and foreign material, including bacterial toxins. The two primary means by which a solute can cross the epithelium are the transcellular route and the paracellular route. The tight junction (TJ) is the most apical of the intercellular junctions and is believed to be the primary rate-limiting barrier to paracellular passage of solutes. (21,22) Occludin, claudins, and junction adhesion molecules (JAMs) form the transmembrane component of the TJ, and result in the sealing of the paracellular space. (14,17,43) Thus far, there have been more than 20 members of the claudin family of proteins identified and it appears that the claudins are responsible for formation of TJ strands and creating the TJ’s physiological barrier. (14,19,25,48)

We are interested in the recovery of intestinal epithelial barrier function. Previously, we have utilized a porcine ileal ischemic-injured model to disrupt barrier function and monitor its recovery in vitro. We have demonstrated that barrier function of ischemic-injured porcine ileum can be rescued by addition of prostaglandins. The response of injured epithelium to prostaglandin E$_2$ (PGE$_2$) is characterized by an immediate, sharp increase in Cl secretion, followed by a gradual increase in transepithelial electrical resistance (TER). (3-5) It is important to note that in this model, PGE$_2$ does not affect epithelial migration (restitution), but appears to restore tight junction structure within restituting epithelium in order to re-establish normal indices of barrier function. Interestingly, this restoration of barrier function is dependent on activation of Cl secretory pathways.

T84 cells are a widely accepted epithelial cell culture model for the study of epithelial chloride secretion (7,30,32) and tight junction function. (33-35,56) One agent previously shown to induce chloride secretion in T84 cells is PGE$_2$. (23,45,54,58) In an effort to
understand the mechanisms by which PGE$_2$ is capable of regulating the TJ barrier we sought to develop a cell culture model to simulate our “leaky” porcine ischemia-injured ileal model. First, hypoxia alone is not sufficient to disrupt the epithelial barrier of T84 cells (20,23) and PGE$_2$ had no effect on restoring barrier function in T84 cells injured by chemical hypoxia (2,11,37), possibly because of residual deficiencies in ATP-dependent signaling mechanisms. Thus, we grew T84 cells to confluence, but studied them at low TER values (200-500 ohms.cm$^2$) when the epithelium would be considered “leaky’ similar to recently restituted epithelium. Our data show that basolateral application of PGE$_2$ (1µM) induces an immediate, sharp, increase in chloride secretion in the low TER model and this increase in chloride secretion precedes improvements in TER. Therefore, we speculated that PGE$_2$-induced chloride secretion is necessary for improvements in barrier function in “leaky” epithelia and that PGE$_2$ induces redistribution of tight junction proteins to the apical lateral membrane in “leaky” epithelia.
**Methods and Procedures**

**Cell Culture**

T84 cells obtained from American Type Culture Collection (Rockville, MD) were grown in a 5% CO₂ humidified incubator at 37°C on 75-cm² flasks (Corning Costar, Acton, MA) with medium containing a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM)/Hams F12 supplemented with 5% heat-inactivated fetal bovine serum, 15 mM HEPES, and penicillin/streptomycin (10µg/mL) at a pH of 7.4. Cells were passaged weekly upon reaching confluence. For experiments, cells were plated onto polyester membrane Snapwell Transwells where they were fed every 2-3 days, and utilized at TER values of 200-500 ohms.cm² or >1200 ohms.cm².

**TER measurements**

T84 cells plated on Snapwell Transwells were placed in Ussing chambers for measurements of short circuit current (Isc) and TER. PGE₂ (1µM) was applied basolaterally after an initial 30 min equilibration period and TER was measured over a 4-hour period. Bumetanide (0.1mM), an inhibitor of the Na⁺/K⁺/2Cl⁻ cotransporter was applied basolaterally prior to addition of PGE₂.

**Triton X-100-soluble and -insoluble fractions**

Triton X-100-soluble and -insoluble fractions have been utilized previously to determine the localization of tight junction proteins.(1,39,40,56) Proteins found in the Triton X-100-insoluble fraction have been associated with the tight junction. T84 monolayers grown on 12mm Transwell inserts were washed with ice-cold PBS and then lysed with a previously
documented(56) 1% Triton X-100-based lysis buffer (1% Triton X-100, 50mM TrisHCl, 50mM EGTA, 30mM sodium pyrophosphate, 50mM NaF, 100µM Na₃VO₄, and complete protease inhibitor cocktail tablets from Roche Molecular Biochemicals, Indianapolis, IN). The supernatant was completely removed and was designated the Triton X-100-soluble fraction. The cells remaining on the filter were solubilized with heated (95°C) 1% SDS-based lysis buffer (1% SDS, 50 TrisHCl, 140 EGTA, 30 sodium pyrophosphate, 50 NaF, 100 µM Na₃VO₄, and complete protease inhibitor cocktail tablets). The cells were scraped from the filter, collected, and heated at 95°C for 5 min followed by brief sonication. This was designated the Triton X-100-insoluble fraction. Protein analysis of extract aliquots was performed (DC protein assay, Bio-Rad, Hercules, CA) and protein concentrations in Triton X-100-soluble and -insoluble fractions were equalized separately.

**Immunoprecipitation**

T84 monolayers grown on 12mm Transwell inserts were treated with PGE₂ (1µM) or bumetanide (0.1mM) for 30 min and followed by washes in ice-cold PBS. Whole cell lysates were extracted with lysis buffer containing (in mM) 50 NaCl, 50 Tris, 0.5% Na Deoxycholate, 1% Triton-X 100, 5 EGTA, 1 Na₃VO₄, 1 PMSF, 1 NaF, 1µg/mL aprotinin, 1µg/mL leupeptin. Samples were normalized to a concentration of 1.25 mg/ml and were incubated overnight at 4°C in the presence of rabbit anti-claudin-1 antibody. Immune complexes were precipitated with protein A/G PLUS agarose beads for 2 hours and washed three times.
**Gel electrophoresis and Western blotting**

Samples were mixed with an equal volume of 2x SDS-PAGE sample buffer and boiled for 4-minutes at 100°C. Lysates were loaded on a 10% SDS-polyacrylamide gel and electrophoresis was carried out according to standard protocols. Proteins were transferred to a nitrocellulose membrane (Hybond ECL, Amersham Life Science, Birmingham, UK) using an electroblotting mini-transfer apparatus according to the manufacturer’s protocol. Membranes were blocked in Tris buffered saline (TBS) containing 0.05% Tween (TBS-T) and 5% dry powered milk. Membranes were washed three times for 5-minutes each with (TBS-T) and incubated for 2 hr at room temperature in primary antibody. Overnight incubations in TBS-T with 1% BSA were performed for the phospho-tyrosine antibody. After washing three times for 5-minutes each with TBS-T, the membranes were incubated for one hour with horseradish peroxidase conjugated secondary antibody. After washing two times for 5-minutes each with TBS-T and one time with TBS for 15-minutes, the membranes were developed for visualization of protein by addition of enhanced chemiluminescence reagent (Amersham, Princeton NJ).

**Immunofluorescence**

T84 cells were fixed with ice-cold 100% methanol for 20 min at –20°C followed by 2 washes with cold PBS and blocked for 30 min in PBS plus 1% BSA. This was followed by incubation with 10µg/mL of anti-claudin-1 for 45 min, 2 washes with cold PBS, and incubation with Alexa 488 tagged anti-rabbit mouse IgG from Molecular Probes. Two washes were completed with cold PBS and filters were mounted on slides utilizing Molecular Probes Prolong Antifade solution. Images were captured on a Leica Laser Scanning Confocal...
Microscope with the assistance of Dr. Eva Johannes, Department of Botany, North Carolina State University.

**Materials**

Transwell inserts were purchased from Corning Costar. PGE$_2$ was obtained from Sigma (St. Louis, MO). Tissue culture reagents were purchased from Mediatech/Cellgro (Herndon, VA). Antibodies to occludin, claudin-1, -3, and -5 were purchased from Zymed (San Francisco, CA). Anti-phosphotyrosine antibody (clone 4G10) was purchased from Upstate Biotechnology (Lake Placid, NY) and anti-phosphothreonine was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Statistical analysis**

All data were analyzed using a statistical software package (Sigmastat, Jandel Scientific, San Rafael, CA). Data were reported as mean ± standard error of the mean (SE) for a given number (‘n’) for each experiment. Results were analyzed by 2-way ANOVA on repeated measures. For analyses where significance was detected by ANOVA, the Tukey’s test was utilized for post-hoc pairwise multiple comparisons. The $\alpha$ level for statistical significance was set at $p<0.05$. Where a significant time x treatment interaction was noted, a one-way ANOVA was utilized to differentiate individual treatment effects.
Results

The effect of PGE$_2$ on Isc and TER

Our first effort was devoted to developing an intestinal epithelial cell culture model to simulate our porcine model for evaluation of barrier function recovery. Initially, we attempted to utilize a previously characterized chemical hypoxia model in T84 cells for disruption of barrier function.\(^{(2,11,37)}\) Although chemical hypoxia was capable of disrupting barrier function in T84 cells, the administration of PGE$_2$ (1µM) had no effect on rescuing TER (Fig. 1). We also examined the literature to determine if hypoxia alone would be a suitable model to disrupt barrier function and simulate our porcine model. Previously, it has been shown that up to 72-hours of absolute hypoxia is not capable of disrupting barrier function.\(^{(20,23)}\) Thus, our next effort was to examine the T84 cells at a low TER (200-500 ohms.cm$^2$). Typically, T84 cells are utilized at TER values of >1000 ohms.cm$^2$.\(^{(32,34,45,49)}\) We speculated that T84 cells grown to low TER values would simulate our porcine model of “leaky” epithelia. As seen in Figure 2, T84 cells examined at low TER values (200-500 ohms.cm$^2$) responded to basolateral administration of PGE$_2$ with an immediate, sharp, increase in Isc (Fig. 2A) followed by a significant (p<0.05) and prolonged increase in TER (Fig. 2B). Furthermore, basolateral administration of bumetanide (0.1mM), an inhibitor of the basolateral Na$^+$/K$^+$/2Cl$^-$ cotransporter, prevented the PGE$_2$-elicited increase in Isc and TER. We followed this study with high TER (>1200 ohms.cm$^2$) T84 cells to confirm that PGE$_2$ elicited increases in Isc result in decreases in TER, as previously reported.\(^{(54)}\) Figure 3 shows that in high TER T84 cells, PGE$_2$ (1µM) results in a sharp, immediate rise in Isc (Fig 3A), followed by a decrease in TER values (Fig. 3B). Thus, it is evident that T84 cells respond quite differently depending upon their baseline level of “leakiness.”
The effect of PGE$_2$ on tight junction proteins

After observing an increase in TER upon basolateral administration of PGE$_2$, we sought to determine the effects of PGE$_2$ on specific TJ proteins. Initially, we examined Triton-X-100 solubility fractions of several TJ proteins. Triton-X-100 solubility and insolubility has been previously utilized to distinguish between TJ proteins associated with the membrane or cytoplasm, respectively.\textsuperscript{(44,47,56)} As seen in Figure 4A, there was no change in claudin-1 distribution between cell fractions after two hours in any of the treatment groups. Furthermore, there were no changes in Triton-X-solubility of claudin-3 (Fig 4B). Additionally, there were no differences in claudin-5 or occludin cell fraction distribution between treatment groups (data not shown). Next, we performed the same Triton-X-100 solubility analysis at four hours. Interestingly, PGE$_2$ induced a shift in claudin-1 from the Triton-X-100 soluble fraction to the Triton-X-100 insoluble fraction (Fig. 5). Furthermore, pre-treatment of cells with bumetanide prevented the PGE$_2$-induced shift. However, after four hours, there were still no treatment-induced changes in claudins-3, -5, or occludin distribution (data not shown). To verify that the Triton-X-100 solubility method was indicative of the location of the TJ proteins, we performed immunoflourescence analyses. Immunoflourescence for claudin-1 supported our Triton-X-100 solubility Western Blots. Figure 6, shows that PGE$_2$ enhanced immunoflourescence of claudin-1 at the region of the tight junction when compared to untreated cells. Additionally, pre-treatment with bumetanide prevented the PGE$_2$-elicited effects on claudin-1 distribution.

The effect of PGE$_2$ on phosphorylation of claudin-1
Our next effort was to determine a possible means by which PGE$_2$ could induce early changes in TER noted in our initial experiments. We therefore attempted to determine if PGE$_2$ induced phosphorylation of claudin-1 as an indication of an early signaling event in restoration of tight junctions. We treated the low TER (200-500 ohms.cm$^2$) T84 cells for 30 minutes with PGE$_2$ (1µM), bumetanide (0.1mM), or a combination of these agents. We then extracted whole cell lysate proteins, immunoprecipitated claudin-1, and performed a Western Blot for phospho-threonine or phospho-tyrosine. We observed evidence of claudin-1 threonine phosphorylation, but there were no effect of any of our treatments on the level of phospho-threonine claudin-1 expression (Fig. 7A). Conversely, PGE$_2$ (1µM) induced an upregulation in the amount of phospho-tyrosine (Fig. 7B) and this PGE$_2$-elicited effect was prevented by administration of bumetanide (0.1mM).
**Discussion**

Utilizing a porcine ileal ischemia-injured model to disrupt barrier function and monitor mucosal recovery in vitro, we have previously demonstrated that PGE₂-elicited chloride secretion precedes restoration of barrier function. (3-5) However, the degree to which we can use the porcine model to study mechanisms by which PGE₂ restores mucosal barrier function is limited by the complexity of the tissue, which contains a broad range of cell types. In order to focus on epithelial events, our current work, presented herein, demonstrates that the human colonic epithelial cell line, T84, can be utilized to mimic PGE₂-mediated reparative events first documented in our porcine model. In particular, we had shown in porcine tissues that increases in TER are preceded by increases in Isc, associated with changes in paracellular architecture within restituting epithelium. (3-5) Utilizing confluent T84 cells at a TER between 200-500 ohms.cm² to simulate “leaky” epithelia, we have confirmed that PGE₂-induced increases in Isc, indicative of chloride secretion, precede and are necessary for improvements in barrier function, as measured by TER. However, we have been able to conclusively show that these changes in epithelial transport are directly associated with changes in tight junction structure. In particular, PGE₂ induced redistribution of claudin-1 to the apical lateral membrane, an effect that was abolished by bumetanide, an inhibitor of the basolateral Na⁺/K⁺/2Cl⁻ cotransporter. Furthermore, we demonstrated that PGE₂ induces tyrosine phosphorylation of claudin-1, which precedes observed structural changes in claudin-1. To our knowledge this is the first report that tyrosine phosphorylation of claudin-1 is mechanistically linked to changes in TJ barrier function in intestinal epithelial cells.
T84 cells have been extensively utilized to examine intestinal epithelial chloride secretion.(31,32,50,52,54) Additionally, T84 cells appear to be a good model cell line for studying regulation of the TJ in intestinal epithelium.(6,26,53,56,57) Here we demonstrate that dependent upon the level of “leakiness” of the barrier, intestinal epithelium responds quite differently to the chloride secretagogue, PGE2. Induction of chloride secretion has previously been reported to be associated with decreased barrier function.(36,54) Our data agrees with this prior work by showing that induction of chloride secretion, via basolateral PGE2 administration, can result in a loss of barrier function in high TER (>1200 ohms.cm²) T84 cells. Presumably, this is due to a loss of apical membrane conductance and is a transcellular effect. Similarly, basolateral application of PGE2 in low TER (200-500 ohms/cm²) T84 cells, simulating “leaky” epithelia, results in chloride secretion. However, in contrast to high TER cells, the “leaky” epithelia respond with an improvement in barrier function, as measured by TER. We believe this effect to be paracellular in nature, rather than transcellular. This speculation is supported by prior work demonstrating that in low resistance epithelia, transcellular resistance is several orders of magnitude higher than paracellular resistance. Because of the dominant effect of the low resistance pathway in leaky epithelia, changes in TER are associated with changes in paracellular resistance.(8)

Paracellular pathways have ion selectivity and claudins are the major determinant of paracellular ion selectivity and barrier function.(9,10,15,51) Thus, regulation of claudins by paracrine mediators such as prostanoids could have meaningful physiologic implications. PGE2 is released under various inflammatory conditions,(41,42,46) yet its pathophysiologic role remains unclear. Our data suggests that PGE2 is capable of enhancing barrier function via tyrosine phosphorylation and apical membrane localization of the TJ protein claudin-1.
Phosphorylation of claudins has been previously reported in MDCK cells (55), rat lung endothelial cells, (13) and blood brain barrier endothelial cells. (28) Furthermore, the phosphorylation of claudin-1 in MDCK cells has been shown to be associated with increased Cl\textsuperscript{−} permeability. (55) We speculate that PGE\textsubscript{2} enhanced phosphorylation of claudin-1 denotes a regulatory change of claudin-1 which precedes and is necessary for localization of claudin-1 to the region of the TJ. We believe that regulation of claudin-1 via phosphorylation status could play a role in targeting claudin-1 to the apical lateral membrane. Supporting our speculation is previous work demonstrating that the C-terminal sequence of all claudins share a YV motif in the final two amino acids that is required for binding to the ZO proteins (29,38). Thus, we speculate that tyrosine phosphorylation of claudin-1 could be a regulatory mechanism targeting this protein to the membrane. Similarly, additional work has shown that occludin, another protein associated with the TJ, can be phosphorylated at sites within the C-terminus, resulting in enhanced barrier function. The interaction of the C-terminal cytoplasmic domain with structural proteins, such as ZO-1, may serve to link occludin to the actin cytoskeleton. (12,18) Furthermore, ZO-2 and –3 also bind to the C-terminus of occludin. (24,29)

We have also demonstrated that PGE\textsubscript{2} induces structural changes in claudin-1 as analyzed by Triton-X-100 solubility and immunofluorescence. The PGE\textsubscript{2}-induced improvements in TER appear to be associated with localization of claudin-1 to the region of the TJ. Thus, we speculate that PGE\textsubscript{2} initiated enhancement of barrier function in “leaky” epithelia is a result of claudin-1 localization to the TJ. Supporting our work is a report that over expression of claudin-1 in MDCK cells results in a 4-fold increase in TER and decreased flux of 4kDa dextran when compared to control cells. (27) Furthermore, the
importance of claudin-1 has been demonstrated in mice, as claudin-1 deficient mice died within one day of birth due to transepidermal water loss. Thus, it appears that claudin-1 is critical to epithelial barrier function.

In conclusion, we have shown here that PGE$_2$ induced chloride secretion appears to initiate tyrosine phosphorylation of claudin-1 and its localization at the region of the TJ in “leaky” epithelia. We speculate that regulation of claudin-1 via PGE$_2$ induced tyrosine phosphorylation is necessary for targeting claudin-1 to the region of the TJ, which ultimately results in enhanced intestinal barrier function. This speculation is supported by previous work showing that claudin-1 has a YV motif, which is required for binding to ZO proteins. Future work will focus on determining which tyrosine residue is phosphorylated and its role in localizing claudin-1 to the region of the TJ.


Figure 1: The effect of chemical hypoxia on TER in T84 cells. Chemical hypoxia was induced 30 min after mounting T84 cells in Ussing chambers. At 60 min (30 min after initiation of chemical hypoxia), the chemical hypoxia solution was removed and replaced with normal ringers solution. Immediately after washout of the chemical hypoxia solution, PGE\(_2\) (1\(\mu\)M) was added to the basolateral surface of select cells. PGE\(_2\) had no effect on enhancing recovery of TER after chemical hypoxia. Although not statistically significant, PGE\(_2\) slightly inhibited recovery of TER after chemical hypoxia. Values represent means ± SE, n=4

Figure 2: The effect of PGE\(_2\) on Isc and TER in “leaky” T84 cells. Fig 2A demonstrates that addition of 1\(\mu\)M PGE\(_2\) to the basolateral surface of “leaky” T84 cells induces an immediate, sharp, and significant (p<0.05) increase in Isc. Application of bumetanide, an inhibitor of the basolateral Na\(^+\)/K\(^+\)/2Cl\(^–\) cotransporter prevents the PGE\(_2\) induced effect on Isc. Fig 2B demonstrates that 1\(\mu\)M PGE\(_2\) applied to the basolateral surface of “leaky” T84 cells results in a significant (p<0.05) enhancement of TER. Bumetanide also prevented the PGE\(_2\) induced enhancement in TER. Values represent means ± SE, n=4

Figure 3: The effect of PGE\(_2\) on Isc and TER in high TER T84 cells. Fig 3A demonstrates that addition of 1\(\mu\)M PGE\(_2\) to the basolateral surface of T84 cells induces an immediate, sharp, and significant (p<0.05) increase in Isc. Fig 2B demonstrates that 1\(\mu\)M PGE\(_2\) applied to the basolateral surface of T84 cells results in a significant (p<0.05) reduction in TER. Values represent means ± SE, n=4
Figure 4: The effect of PGE$_2$ and bumetanide on Triton-X 100 solubility of claudins-1 and –3 after 2 hours of treatment. Fig 4A shows that PGE$_2$ and bumetanide were without effect on claudin-1 Triton-X 100 solubility after 2 hours. Fig 4B shows that PGE$_2$ and bumetanide were without effect on claudin-3 Triton-X 100 solubility after 2 hours. One representative blot of 3

Figure 5: The effect of PGE$_2$ and bumetanide on Triton-X 100 solubility of claudin-1 after 4 hours of treatment. Fig 5 shows that PGE$_2$ induced a shift in the amount of claudin-1 in the Triton-X 100 insoluble fraction. Application of bumetanide prevented the amount of claudin-1 in the PGE$_2$ treated Triton-X 100 insoluble fraction. One representative blot of 3

Figure 6: Immunofluorescence analysis of claudin-1 at 4 hours after treatment. PGE$_2$ induced an accumulation of claudin-1 at the region of the TJ when compared to untreated, control cells and cells treated with bumetanide. Bumetanide inhibited the PGE$_2$ induced effects on claudin-1 distribution.

Figure 7: Western blot analysis for phosphorylated tyrosine and threonine residues on claudin-1. T84 cells were treated for 30 min, followed by extraction of proteins, immunoprecipitation for claudin-1, and blotting for phospho-threonine or phospho-tyrosine. Fig 7A shows that the level of phospho-threonine was unaltered in each treatment group. Fig 7B shows that PGE$_2$ treatment resulted in an enhanced amount of phospho-tyrosine when compared to control cells or those treated with bumetanide. The levels of phospho-tyrosine in
the PGE$_2$ treated cells were similar to those treated with activated pervanadate (labeled as “+ cont”), a tyrosine phosphatase inhibitor. One representative blot of 3
Shifflett et al. Figure 1:

[Graph showing time (min) and % change in TER with different conditions: Control, ATP deplete/replete, ATP deplete/replete + PGE\(_2\) (1\(\mu\)M), with annotations for Chemical hypoxia and Washout.]
Shifflett et al. Figure 2

A

- Control
- PGE$_2$ (1μM)
- Bumetanide (0.1mM)
- Bumetanide + PGE$_2$

Change in Isc

Time (min)

0 15 30 45 60 75 90 105 120 135 150

B

- Control
- PGE$_2$ (1μM)
- Bumetanide (0.1mM)
- Bumetanide + PGE$_2$

% Change in TER

PGE$_2$ and Bumetanide addition

Time (min)

0 30 60 90 120 150 180 210 240
Shifflett et al. Figure 3

A

- Control
- PGE₂ (1μM)

% Change in Isc

Time (min)

0 25 50 75 100 125 150

B

- Control
- PGE₂ (1μM)

% Change in TER

Time (min)

0 25 50 75 100 125 150
Shifflett et al. Figure 4A

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Claudin-1

* Samples at 2 hours

Shifflett et al. Figure 4B

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Claudin-3
Shifflett et al. Figure 5

Triton-X Soluble

Cont  PGE2  Bumet

Triton-X Insoluble

Cont  PGE2  Bumet

Claudin-1

* Samples at 4 hours
Shifflett et al. Figure 7A

Shifflett et al. Figure 7B