ABSTRACT

LITTLE, DIANNE. Pathways of Prostaglandin-Mediated Recovery of Ischemia-Injured Intestine. (Under the direction of Anthony Thomas Blikslager.)

Colic is the leading cause of death in horses after old age. Many of these deaths are attributable to the effects of absorption of endotoxin through a compromised intestinal barrier and the systemic effects of endotoxemia. Many of the effects of endotoxemia are mediated by cyclooxygenase (COX) elaborated prostaglandins (PGs). Horses with colic are commonly treated with non-steroidal anti-inflammatory drugs (NSAIDs), such as flunixin meglumine which inhibit cyclooxygenase and therefore inhibit prostanoid production. However, we have previously shown that PGs are required for recovery of intestinal barrier function after ischemic-injury. Administration of non-selective COX inhibitors such as flunixin meglumine inhibit PG production and therefore retard recovery of intestinal barrier function in the horse after ischemic-injury.

The aims of this work were firstly to evaluate the intra-cellular signaling pathways involved in this PG-mediated recovery using a porcine model of intestinal ischemia, and secondly to use an equine model of intestinal ischemia to determine if the NSAID meloxicam would permit PG-mediated recovery of intestinal barrier function after ischemic-injury. We found that a luminal osmotic gradient generated by PG-mediated chloride secretion signals via phosphatidylinositol-3-kinase (PI3’K) to close the lateral intercellular space and the intercellular tight junction. Furthermore, PI3’K signaling permits PG-mediated redistribution of the tight junction proteins zonula occludens-1 and
occludin back to the tight junction after ischemic-injury. We found that the downstream effector of the small GTPase Rho, Rho kinase (ROCK) was also critical for PG-mediated recovery of barrier function, as was protein kinase C delta (PKCδ). PGs also caused upregulation of PKCδ expression in ischemia-injured mucosa, an effect that was partially mediated by ROCK.

We found in equine studies that the COX-2 preferential inhibitor meloxicam permitted recovery of ischemia-injured equine jejunum to a similar degree as horses not treated with NSAIDs. In contrast, flunixin retarded recovery of intestinal barrier function after ischemic-injury and caused increased flux of lipopolysaccharide in ischemia-injured jejunal mucosa, compared to horses that were not treated with flunixin. Meloxicam provided comparable analgesia and beneficial effects on clinical parameters as flunixin. Therefore meloxicam may be beneficial in treatment of colic in the horse, provided further studies are conducted.
PATHWAYS OF PROSTAGLANDIN-MEDIATED RECOVERY OF ISCHEMIA-INJURED INTESTINE

by

DIANNE LITTLE

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

PHYSIOLOGY

Raleigh
2006

APPROVED BY:

Anthony T. Blikslager    Samuel L. Jones
(Committee chair)

Mat P. Gerard            Jody L. Gookin
For my father
BIOGRAPHY

Dianne Little BVSc MSpVM MRCVS

Diplomate, American College of Veterinary Surgeons

**Education/ Training**

<table>
<thead>
<tr>
<th>Institution and Location</th>
<th>Dates</th>
<th>Degree (Field of Study)</th>
</tr>
</thead>
<tbody>
<tr>
<td>University of Liverpool, England</td>
<td>1993-1998</td>
<td>BVSc with Distinction (Veterinary Science)</td>
</tr>
<tr>
<td>North Carolina State University, Raleigh, NC, USA</td>
<td>2000-2003</td>
<td>MSpVM (Specialized Veterinary Medicine)</td>
</tr>
</tbody>
</table>

**Professional Positions**

- Clinical Fellow, Large Animal Surgery, Oregon State University 1999-2000
- Large Animal Surgery Resident, North Carolina State University 2000-2003
- Research Associate Gastrointestinal Physiology, NCSU 2003-present

**Professional Awards**

- Queen Mother Travel Scholarship, British Equine Veterinary Association Trust: 1997
- Diplomate, American College of Veterinary Surgeons: 2004
- Leroy Coggins Comparative Biomedical Sciences Graduate Student Award: 2005
- NIH Center for Gastrointestinal Biology and Disease Annual NCSU Research Competition: 2005
Publications (Refereed Journals)


**Book Chapters**  

Presentations (National and International Symposia)


Little D. Clinical examination of the New World Camelid. Southeastern Veterinary Conference, Myrtle Beach, SC, June 2001.
ACKNOWLEDGMENTS

I would like to thank Dr. Anthony Blikslager for his professional and personal advice and mentorship over the last nine years. Without his guidance and sense of humor, the path would have been much more difficult. In addition, I would like to thank my committee members Drs. Sam Jones, Mat Gerard and Jody Gookin for their advice, and for being there for me. The example that all of these individuals set will be carried with me throughout my career.

Many of the experiments in this dissertation would not have been possible without the assistance of Donna Hardin, Terri Lucas and Meghan Kruse of the Central Procedures Laboratory. In addition, the help of lab technicians Karen Young, Jenna Wooten, and Kathleen Ryan were all crucial to the smooth-running and completion of these studies. Thanks must also go to Dr. Nigel Campbell and fellow graduate students Adam Moeser and Dr. Vanessa Cook; without their moral support, friendship, help and advice, life as a PhD student would have been much harder.

I acknowledge funding from the American Quarter Horse Association, the National Institute of Health, and the United States Department of Agriculture for the studies within this dissertation, and funding from the British Equine Veterinary Association Trust Queen Mother Travel Scholarship which allowed me to ‘cross the pond’ and experience the opportunities available at the College of Veterinary Medicine at North Carolina State University while I was still a veterinary student.
I wish to thank Professor “Prof” Barrie Edwards C.B.E. and Professor Derek Knottenbelt O.B.E. from the Faculty of Veterinary Science at the University of Liverpool. In 1989, before I had even gained a place at veterinary school, I heard each of them speak at equine client meetings organized by David and Helen Franklin of St.Georges’ Veterinary Clinic, Wolverhampton, England and knew from listening to them that I wanted to follow a career in equine surgery and research. Without their quest for new knowledge, enthusiasm for teaching and clinical cases, passion for veterinary medicine, their inspiration, and their example, I would never have embarked on this path. David and Helen Franklin are great friends and I would not be where I am today without their friendship and support over the years. Chris Rea, Liam Kearns, Graham Potts and Andrew Harrison of the Three Counties Veterinary Hospital, Tewkesbury, Gloucestershire, England all helped mould me into an equine veterinarian, during my years studying at the University of Liverpool.

I would not be here without my father, Gordon, who instilled into me at an early age the values of personal discipline and work ethic. While I was growing up, as I saw how much he cared for his patients and their families, I gained important insights into bedside manner and patient care, which I have tried to emulate. John Metz has been there for me since we met, and has taught me more about life outside of work than anyone. There are numerous other family members and friends who have contributed to my professional and personal development. Without the help and support of so many people, I would not have got this far. Thank you.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>List</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiv</td>
</tr>
</tbody>
</table>

## CHAPTER 1.

The Role of Rho, Protein Kinase C and Phosphatidylinositol-3-kinase in Function of the Tight Junction.

Introduction ................................................................. 1

Rho/Rho Kinase ................................................................. 3

Protein Kinase C ............................................................... 6

Phosphatidylinositol-3-kinase ......................... 15

References ................................................................. 21

## CHAPTER 2.

Phosphatidylinositol-3-Kinase (PI3’K) Signaling is Required for Prostaglandin Induced Mucosal Recovery in Ischemia-Injured Porcine Ileum.

Introduction ................................................................. 37

Materials and Methods ...................................................... 39

Results ................................................................. 45

Discussion ................................................................. 51

References ................................................................. 55
CHAPTER 3.
The Role of Rho Kinase and Protein Kinase C in Recovery of Barrier Function in Ischemia-Injured Porcine Ileum

Introduction ......................................................... 85
Materials and Methods ........................................... 87
Results ............................................................... 90
Discussion .......................................................... 94
References .......................................................... 98

CHAPTER 4.
Cyclooxygenase and its Inhibitors – an Evolving Field

History of cyclooxygenase discovery ............................ 113
Patterns of cyclooxygenase expression ......................... 114
Non-steroidal anti-inflammatory drugs: cyclooxygenase inhibition .......... 117
Non-steroidal anti-inflammatory drugs: cyclooxygenase independent effects...
Phosphatidylinositol-3-kinase (PI3’K)/Akt signaling .............. 119
Uncoupling of oxidative phosphorylation ...................... 122
Peroxisome proliferator-activated receptor activity ................. 124
NFκB activity .......................................................... 128
The mitogen activated protein kinases (MAPK) .................... 130
The heat shock protein response .................................. 132
References .......................................................... 134
CHAPTER 5.

The Effects of the Cyclooxygenase Inhibitors Meloxicam and Flunixin Meglumine on Recovery of Ischemic-Injured Equine Jejunum. .......................... 154

Introduction ................................................................. 155

Materials and Methods .................................................. 156

Results ............................................................................. 165

Discussion ...................................................................... 170

Acknowledgments .......................................................... 184

Footnotes ........................................................................ 184

References ....................................................................... 185
LIST OF TABLES

CHAPTER 1.

1. PKC Isoforms, Second Messengers, Cofactors, Activators and Inhibitors… 32
2. Classes, Composition, Substrates and Products of Different PI3’K Isoforms. 35

CHAPTER 2.

1. Morphometric Assessment of Epithelial Restitution ............................. 60

CHAPTER 5.

1. Behavioral Pain Scoring System. ................................................... 192
2. Summary of Noncompartmental Pharmacokinetic Parameters Derived Following IV Administration of Meloxicam. ................................. 202
LIST OF FIGURES

CHAPTER 1.

1. Diagram of Activation and Inactivation of Rho………………………… 31
2. Schematic Representing Epithelial Chloride Secretion………………… 33
3. Schematic Summarizing Major Effects of PKC Isoforms on the Tight Junction…………………………………………………………………………………… 34

CHAPTER 2.

1. Electrical Responses of Ischemia-Injured Tissues Treated with Indomethacin, 16,16-Dimethyl Prostaglandin E2 and Carbacyclin……… 61
2. Histologic Appearance of Ischemia-Injured Porcine Ileal Mucosa……… 63
3. Evaluation of the Effects of Indomethacin and Prostaglandins on Serosal-to-Mucosal Fluxes of $^{22}$Na, and Mucosal-to-Serosal Fluxes of $^3$H-mannitol and $^{14}$C-inulin……………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………xiv
10. Electrical Responses of Tissues to Inhibition of Phosphatidylinositol-3-kinase (PI3’K) with LY294002. ................................................. 76
11. Effect of inhibition of Phosphatidylinositol-3-kinase (PI3’K) on urea-induced recovery. ................................................................. 78
12. Immunoblots for Tight Junction Proteins...................................... 79
13. Immunofluorescence microscopic evaluation of ischemia-injured tissues for occludin. ................................................................. 80
14. Immunofluorescence Microscopic Evaluation of Ischemia-Injured Tissues for ZO-1 ................................................................. 81
15. Proposed Model of Prostaglandin Signaling Pathways................... 82

CHAPTER 3.

1. Treatment with the Rho Inhibitor Clostridium difficile Toxin A had no Effect on PG Induced Recovery of Barrier Function............... 102
2. The Specific Rho Kinase Inhibitor Y27632 Significantly Inhibited PG Induced Recovery of Barrier Function, with no Effect on Isc. ...... 103
3. TGFβ1 had no Significant Effect on PG Mediated Recovery of TER but Reduced the Increase in Isc at 0.5ng/ml ............................... 104
4. HSP72 Expression in Control and Ischemia-Injured Tissues............. 105
5. Treatment with the Non-specific PKC Inhibitors Tamoxifen and Staurosporine does not block PG-Induced Recovery.................... 106
6. The Selective PKCδ Inhibitor Rottlerin Partially Blocked Recovery of Barrier Function Stimulated by PGs. .......................... 107

7. Trend Toward Blockade of Recovery of Barrier Function with the
PKCα,β,γ,δ inhibitor Gö6983......................................................... 108

8. No Significant Effect of the PKCα and β1 inhibitor Gö6976 on
Recovery of Barrier Function. ................................................. 109

9. Expression of Total PKC ......................................................... 110

10. Expression of ROKα/ROCK II ............................................ 111

CHAPTER 4.

1. Pathways of Arachidonic Acid Metabolism.............................. 149

2. Interaction of the Phosphatidylinositol-3-Kinase Pathway with the NFκB Pathway via Akt............................................................. 150

3. PPARγ Activation Blocks NFκB Nuclear Translocation .............. 151

4. Recovery of Indomethacin Treated Ischemia-Injured Porcine Ileum and Response to Prostaglandins in the Presence or Absence of the Specific ERK 1/2 Inhibitor PD-98059.................................................. 152

5. Response of Control Tissue Treated with Indomethacin to Specific Inhibition of ERK 1/2 ............................................................... 153
CHAPTER 5.

1. Total Pain Scores for Three Treatment Groups of Six Horses……………….. 193

2. Mean (±SEM) Heart Rate and Respiratory Rate for Three Treatment Groups of Six Horses…………………………………………………………….. 194

3. Mean (±SEM) Transepithelial Electrical Resistance (TER) in Control and Ischemia-Injured Equine Jejunum………………………………………………………………………………….. 195

4. Inulin and LPS Flux across Equine Jejunum over a 1-hour Period in the Ussing Chamber…………………………………………………………….. 196

5. Numbers of Mucosal Neutrophils in Equine Jejunum………………………… 198

6. Mean (±SEM) percent of Villus Denuded of Epithelial Cells …………………….. 199

7. Mean (±SEM) Villus Height in Control or Ischemia-Injured Jejunum…. 200

8. Plasma Concentrations of Meloxicam ………………………………………….. 203

9. Western Blot Analysis of Control and Ischemia-Injured Equine Jejunum.. 204

10. Densitometry Analysis for COX-1, COX-2, PPARγ, Total P38 and the ratio of Phospho-P38:Total P38…………………………………………………………….. 205
Chapter 1

The Role of Rho, Protein Kinase C and Phosphatidylinositol-3-kinase in Function of the Tight Junction.

Dianne Little
Introduction

The ability to regulate tight junction permeability and repair after injury is critical for maintenance and recovery of intestinal barrier function after injury. The tight junction itself is located in the most apical compartment of the intercellular junctional complex, which also includes adherens junctions, gap junctions and desmosomes. The tight junction is composed of over 40 different proteins. Of these, three protein groups have extracellular domains, occludin, junction adhesion molecules (JAMs) and the claudins, of which 24 have been identified (Van Ittalie and Anderson, 2006). The exact claudin expression profile of a tight junction depends on numerous factors including species, anatomical site, and local permeability requirements (Van Ittalie and Anderson, 2006). Occludin is thought to be primarily responsible for cell signaling rather than formation of the tight junction barrier, (Barrios-Rodiles et al. 2005) whereas the claudin expression profile of a tight junction is responsible for charge and size selectivity of the junction (Van Ittalie and Anderson, 2006). Other proteins such as ZO-1, -2 and -3 physically interact with these 3 transmembrane proteins, and ultimately link to the actin cytoskeleton, which at its most simplistic contracts to open the tight junction, and relaxes to close it. A wide variety of signaling molecules are involved in regulation of the tight junction, either through interaction with the actin cytoskeleton or via direct interaction with the proteins that form the tight junction.
Rho/Rho kinase

The Rho family in mammals contains 19 small GTP-binding proteins that are critical for regulation of the cytoskeleton in response to external signals (Takai et al. 2001). Study of their function has been facilitated by the use of specific Clostridial toxins. Clostridium botulinum transferase C3 causes ADP-ribosylation of Rho A proteins, Clostridium difficile Toxin A (CdTA) and B (CdTB) both cause glucosylation of Rho, thus preventing interaction with downstream effectors. These toxins all inhibit Rho, in contrast to Escherichia coli Cytotoxic Necrotizing Factor-1(CNF-1) which causes deamidation of Rho and constitutive activation (Hopkins et al. 2000).

Different members of the Rho family regulate different aspects of the cytoskeleton, for example Rac and cdc 42 regulate formation of lamellipodia, and Rho regulates stress fiber formation between the extracellular matrix and the cell (Takai et al. 2001). However, RhoA is currently identified as the Rho family member of most importance to regulation of tight junction function. Activation and inactivation of Rho is regulated by GTP-ase activating proteins (GAP), Guanine nucleotide exchange factors (GEF) and guanosine nucleotide dissociation inhibitors (GDI) (Takai et al. 2001) (Figure 1). Ezrin, radixin, moesin (ERM), critical proteins involved in the cross-linking of actin filaments to the plasma membrane (Yonemura et al. 2002), can displace GDI from Rho, which is of particular importance in trafficking of tight junction proteins and electrolyte channels to and from the apical membrane (Takai et al. 2001). Down stream targets of Rho A that are critical for regulation of the actin cytoskeleton include protein kinase N, citron kinase,
p21-activated protein kinase, rhophillin and rhotekin and the Rho Kinases (ROKs) (Noma et al. 2006). The ROKs, ROKα/Rho kinase II and ROKβ/ ROK I in turn phosphorylate and inactivate myosin light chain phosphatase and phosphorylation of myosin light chain resulting in myosin ATPase activation resulting in sustained actinomyosin contraction, and phosphorylation of ERM. Other downstream effects of Rho A include PKC, MAP kinase kinase kinase (MAPKKK), actin monomer binding proteins, phosphatidylinositol-3 kinase (PI3’K) and nuclear factor kappa beta (NFkB) (Hopkins et al. 2000, Takai et al. 2001). RhoA mutation in cell lines results in increased paracellular permeability, loss of organization of tight junction transmembrane proteins as seen on freeze-fracture microscopy and redistribution of ZO-1 and occludin away from the tight junction (Jou et al. 1998). In another study, RhoA mutation, or inhibition of ROK II with Y27632 prevented stress fiber formation, myosin light chain phosphorylation, and occludin phosphorylation, leading to reduction in horse-radish peroxidase flux in LPA stimulated ECV304 cells (Hirase et al. 2001). Inhibition of ROK also causes condensation of F-actin away from the perijunctional F-actin rings, and an increase in paracellular permeability without causing redistribution of tight junction proteins away from the junction or their membrane microdomains (Walsh et al. 2001). ROCK does however colocalize with ZO-1 and inhibition of ROK prevents reformation of the TJ after disruption due to failure of the F-actin cytoskeleton to reassemble (Walsh et al. 2001).

*Clostridium difficile* is the cause of life-threatening disease pseudomembranous colitis associated with antibiotic administration, therefore much of the experimental work to
evaluate the role of Rho in regulation of tight junction function has focused on CdTA and CdTB Rho glucosylation (Hopkins et al. 2000). Application of CdTA to confluent T84 intestinal epithelial cells causes an increase in paracellular permeability, caused by disruption of the F-actin in the peri-junctional actinomyosin ring (Hecht et al. 1988). Further studies have found that the increase in paracellular permeability precedes rearrangement of the cytoskeleton. CdTA elevates PKCα/β activity which results in redistribution of ZO-1, occludin, and claudin-1 away from the tight junction. The increase in PKCα/β activity observed in this study preceded Rho A glucosylation, and RhoA glucosylation could be inhibited by PKCα/β inhibition demonstrating a mechanism of action of CdTA that is independent of its effects on Rho A (Chen et al. 2002). RhoA glucosylation by CdTA or CdTB also caused redistribution of ZO-1, ZO-2 and occludin away from the tight junction (Nusrat et al. 2001).

However, it appears that activation or inhibition of RhoA per se is not critical for tight junction assembly, rather an intact on/off molecular ‘switch’ is necessary for the maintenance of barrier function, since constitutive activation of Rho by CNF-1 also resulted in increased paracellular permeability through F-actin and actin binding protein restructuring and redistribution of ZO-1, occludin and JAM-1 away from the tight junction (Hopkins et al. 2003).

The role of RhoA inhibition on function of electrolyte channels important to generation of a luminal osmotic gradient collapse of the lateral intercellular space and closure of the
tight junction has been less well studied. However, CdTB can directly inhibit NHE3, the apical isoform of the electro-neutral sodium hydrogen exchanger, with little effect on the baso-lateral NHE1. The mechanism of this inhibition may be due to altered interaction between NHE3 and the actin cytoskeleton and impaired ability of ezrin to anchor NHE3 to filamentous actin, leading to internalization of NHE3 (Hayashi et al. 2004).

**Protein Kinase C**

The PKC family of serine/threonine kinases is crucial for a variety of cellular functions. Twelve distinct isoforms have been identified (Table 1), divided broadly into conventional, novel and atypical isoenzymes, and each have different sensitivity to activators, inhibitors and co-factors, which are summarized in Table 1. All PKCs consist of a single polypeptide chain containing regulatory N-terminal and catalytic C-terminal domains. While conventional isoforms have domains for binding to diacylglycerol and Ca\(^{2+}\), novel PKC isoforms lack the Ca\(^{2+}\)-binding domain, making them maximally responsive to diacylglycerol or phorbol esters. Human and rodent colonic mucosa appears to express a wide range of PKC isoforms whereas most intestinal cell lines, including the T84 cell line, express primarily cPKC\(\alpha\), nPKC\(\delta\), nPKC\(\varepsilon\) and aPKC\(\zeta\), however some PKC\(\beta\)II, PKC\(\gamma\), PKC\(\eta\), PKC\(\mu\), PKC\(\lambda\)/t are also thought to be expressed by some groups.

The predominant electrolyte driving fluid secretion in the intestine is chloride (Cl\(^{-}\)), and has therefore been the most studied. Furthermore, Cl\(^{-}\) secretion drives the creation of the
osmotic gradient that leads to lateral intercellular space collapse and closure of the tight
junction (Moeser et al. 2004). Therefore in any discussion on the role of PKC in tight
junction function, the effects of PKC on Cl\(^-\) and other electrolyte secretion should be
considered.

Chloride secretion is a well-orchestrated event involving a number of membrane channels
(Figure 2). The Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) has two
membrane domains, each comprising six transmembrane segments, two nucleotide-
binding domains (NBD1 and NBD2), and a regulatory domain (R domain) with potential
sites for phosphorylation by protein kinase A (PKA) and protein kinase C (PKC); PKC
phosphorylation has been demonstrated at S686 and S790 (Riordan et al. 1989). Two
predicted PKC sites are also situated in the distal region of NBD1.

Activation of the CFTR is dependent on phosphorylation of the regulatory domain and on
ATP for activation of the gating function. PKA is the most important mediator of
phosphorylation of human CFTR which occurs at multiple sites on the CFTR with no
individual site being essential for activation (Chappe et al. 2004). PKC enhances human
CFTR activation by PKA by increasing the open probability for the CFTR. PKC is also
capable of activating CFTR at low intracellular Ca\(^{2+}\) concentrations, through facilitating
PKA dependent phosphorylation of CFTR (Li et al. 1989). PKC plays a variable role in
other species and cell types (Chen et al. 2004, Chappe et al. 2004). For example the
response of human CFTR to PKC is approximately 90% of that to PKA, but the response
of Xenopus CFTR to PKC is six times the response to PKA (Chen et al. 2004). This difference in response to PKA or PKC is attributable to the presence of a single PKC phosphorylation consensus site in the regulatory domain in all orthologs of CFTR evaluated, except the human CFTR (Chen et al. 2004). PKCε has been found to contribute to CFTR activation in Calu-3 cells (Leidtke and Cole 1998).

The effect of PKC activators on Cl− secretion is variable depending on the experimental model, and in tissue models, differentiating the effect of different activators and therefore PKC on the epithelial cell or on other cell types is difficult. Much of the work evaluating the role of PKC in Cl− secretion has been done using T84 cell models. Extended activation of PKC by phorbol 12-myristate 13-acetate (PMA) progressively inhibits cAMP regulated Cl− secretion (Matthews et al. 1993). This PMA induced PKC activation results in early activation and translocation of nPKCε, and late activation and translocation of PKCα, but not nPKCδ or aPKCζ to the cell membrane (Matthews et al. 1993). Even though nPKCδ is not translocated to the membrane, activation occurs within 30 minutes of exposure to PMA. In inhibitor studies evaluating cAMP elicited Cl− secretion driven elevations in short circuit current (Isc), inhibition of nPKCε attenuated the effect of PMA on Isc (Song et al. 2001). After stimulation with PMA, nPKCε translocates to the basolateral membrane (Song et al. 2001), suggesting that this isoform is responsible for early stimulation of fluid phase endocytosis (pinocytosis), whereas PKCα is responsible for the later inhibition of fluid phase endocytosis (Song et al. 2002). Peak Isc stimulated by forskolin is inhibited by bryostatin-1, and nPKCε again appears to
be the PKC isoform responsible for this. Conversely, the acetylcholine analogue
carbachol, which activates PKC by increased phospholipid turnover and increased DAG
generation, induces a transient increase in Isc that returns to baseline within 10 minutes,
mediated by nPKCε. When applied to forskolin treated cells, carbachol inhibited the
peak Isc response, and again nPKCε was the isoform found to be responsible (Song et al.
2001). Calcium dependent agonists such as carbachol do not cause Cl− secretion via
elevations in cAMP, and do not activate the CFTR, instead they transiently activate
calcium-activated potassium channels in the basolateral membrane. This results in
hyperpolarization of the cell and apical efflux of Cl−. PMA directly inhibits calcium
induced Cl− secretion by phosphorylation of these basolateral potassium channels
(Reenstra 1993).

In other intestinal cell lines, the response to PMA has been less well studied, and in some
instances found to be contrary to the effect of PMA on T84 cells. For example in H29
cells, PMA induces a transient increase in Isc, but overall the effect of PMA on Isc
appears to be inhibitory (Song et al. 2001).

Apical CFTR Cl− channels are not the only ion channel found to be activated by PKC,
and the effects of PMA on Isc occur very rapidly, whereas down-regulation of CFTR
expression occurs much more slowly (Farokhzad et al. 1998) suggesting that inhibition of
transepithelial Cl− secretion by PMA correlates most closely with inhibition of basolateral
K channels and cAMP stimulated NKCC1 function. At least 10 potential PKC
phosphorylation sites have been identified on NKCC1 (Farokhzad et al. 1999). PMA also reduces the number of functional NKCC1 sites at the basolateral membrane (Farokhzad et al. 1998), an effect mediated by PKCε. The NKCC1 cotransporter is activated by nPKCδ in airway epithelia (Leidtke et al. 1997), but the role of this PKC isoform in NKCC1 activation in the intestine has not been investigated.

PMA has no effect on basal duodenal bicarbonate secretion, but potentiates the effects of cAMP stimulated bicarbonate secretion via PKCε (Tuo et al. 2004). CFTR plays an important role in regulation of bicarbonate secretion in the duodenum, and cAMP regulates epithelial Cl⁻ and HCO₃⁻ secretion by activation of CFTR.

Na/H exchangers (NHE) are activated by PKCα in the presence of estradiol or aldosterone in the distal colonic epithelium, resulting in Na⁺ absorption (Harvey et al. 2002). Conversely, NHE3 is inhibited by a PKCα dependent mechanism in response to elevations in intracellular calcium, and is associated with increased binding of PKCα to NHERF2 (Lee-Kwon et al. 2003). PMA and PKC activation inhibit NHE3 by causing an increase in endocytosis and turnover of the channel (Janecki et al. 1998). PKC also regulates the numbers of Na/glucose cotransporters in the plasma membrane (Wright et al. 1997).

PKC is essential for the de novo genesis of the tight junction and for translocation of ZO-1 from intracellular stores to the tight junction, and its subsequent phosphorylation
PKCζ is the only PKC isoform identified to date that co-localizes with ZO-1 at the tight junction in unstimulated Caco-2 and MDCK cells (Dodane and Kachar 1996), and has been shown to participate in the formation, but not the maintenance of the tight junction. This effect may be facilitated by the interaction of PKCζ and PKCλ with ASIP/PAR-3, PAR-6 and PAR-14 (atypical PKC isotype-specific interacting protein), which is crucial for establishment of cell polarity (Yamanaka et al. 2001). PKCα and PKCδ demonstrate cytoplasmic distribution and PKCβ and nPKCε localize in the nuclear region of both Caco-2 and MDCK cells (Dodane and Kachar 1996). nPKCε is able to translocate to the basolateral membrane, but direct colocalization with the tight junction has not been established.

PMA activation of PKCε results in impairment of tight junctional integrity and causes disassembly of T84 monolayers associated with disruption of peri-junctional F-actin (Hecht et al. 1994, Song et al. 2002). This results in a decrease in transepithelial resistance (TER) that becomes evident only after the inhibition of cAMP induced Cl− secretion (Song et al. 2001). From phorbol ester and specific inhibitor studies, PKCε translocates rapidly to the membrane after PMA exposure, then with prolonged exposure cPKCα and PKCδ are activated. However, it appears that this disruption of tight junctional integrity is mediated predominantly by cPKCα since cPKCα but not PKCδ redistributes to the apical membrane after PMA stimulation (Song et al. 2001). Conversely, the non-phorbol ester PKC agonist bryostatin-1 only transiently and minimally reduces TER, causes membrane translocation of cPKCα later than PMA, and
causes cPKCα down-regulation (Song et al. 2001), by increased ubiquitinization of cPKCα. Carbachol has no effect on TER or on cPKCα, consistent with the postulated role of cPKCα in regulation of barrier function. The same group (Yoo et al. 2003) later found that bryostatin-1 increases TER after treatment of T84 cells with the same dose (100nM) and duration of treatment (4 hours) with bryostatin-1, caused by either nPKCδ or nPKCε. The only apparent differences in the T84 model between the two reports were the source of the T84 cells and the means of measuring TER and Isc. In the study by Yoo et al. (2003), nPKCε was found to be responsible for occludin phosphorylation in both Triton-X soluble (cytoplasmic) and insoluble (membrane and tight junction), and induced a shift in claudin-1 and ZO-2 from cytoplasmic to junctional cell fractions. The reason for the conflicting effects of PKCα and PKCε is unknown, but is probably unrelated to occludin phosphorylation (Yoo et al. 2003). PKCθ is responsible for phosphorylation and membrane distribution of claudin-1 and -4 in Caco-2 monolayers (Banan et al. 2005), and is responsible for assembly of the cytoskeleton (Banan et al. 2004).

In insects, nPKCε is a MARCKS kinase and F-actin can act as an isoenzyme-selective receptor for activated C kinase for PKCε (Cabell et al. 1996), to date binding of other PKC isoforms to F-actin has not been demonstrated. The differential effects of different PKC isoforms are likely achieved by translocation of the isoforms to distinct intracellular targets. Tethering of activated PKC at their target involves anchoring proteins, such as RACKs (receptors of activated C-kinase). F-actin has been shown to be a PKCε-specific
RACK, and PKCε has an actin binding site. Binding of diacylglycerol or phorbol esters to PKCε triggers association with actin, and binding of actin filaments maintains the isoform in its active state (Akita 2002).

PKC isoforms are known to decrease phosphorylation of MLC and increase phosphorylation of MLCK (Turner et al. 1999), leading to speculation that this may be the mechanism of PKCα action on tight junction disruption. Some Caco-2 clones and T84 cells demonstrate a progressive decrease in TER in response to PMA, but other Caco-2 clones have been identified that experience increased MLCK phosphorylation and decreased MLC phosphorylation in response to PMA leading to an increase in TER (Song et al. 2001).

In models evaluating the effect of bacteria and bacterial toxins on tight junction disruption in cell monolayers, PKCζ co-localization to occludin in the tight junction occurs with enteropathogenic E. coli (EPEC) infection and but does not appear to contribute to disruption of the tight junction. PKCζ activation however, is substantial with EPEC infection, but is only minimal after enterohemorrhagic E. coli (EHEC) infection. EPEC infection acts through MLCK activation to cause disruption of barrier function, and there is evidence to suggest that PKCζ acts proximally in the MLCK induced reduction of TER (Tomson et al. 2004). Clostridium difficile Toxin A elevates PKCα/β activity and causes PKC mediated RhoA glucosylation and ZO-1, but not ZO-2 translocation away from the tight junction (Chen et al. 2002). Toll-like receptor (TLR)
activation, essential for the recognition and response to microbes, causes PKCα and PKCδ activation, tightening and sealing of tight junction associated ZO-1 and activation of MARCKS (myristoylated alanine-rich C kinase substrate), which besides being a major cellular target of PKC is also an actin cross-linking protein (Cario et al. 2004).

In oxidant-induced injury models, increased PKCδ translocation causes increased expression of inducible nitric oxide synthase and production of nitric oxide, which results in disruption of the cytoskeleton and increased paracellular permeability (Banan et al. 2002, Banan et al. 2003). However, this work evaluated the role of PKCδ in disruption of the tight junction in response to injury, and not in recovery of the tight junction after injury. We have found that PKCδ activity is required for maximal PG-mediated elevation in TER in porcine indomethacin-ischemia injured ileum, and that PKCδ expression is increased in indomethacin-ischemia injured tissue when additionally treated with PGs.

To summarize, a wide range of different PKC isoforms mediate epithelial cell secretion and tight junction function, and depending on the model involved, activation or inhibition of a specific isoform may cause increased or decreased permeability or tight junction assembly or disassembly. The specific isoform involved appears to be very sensitive to the specific intracellular conditions, the duration those conditions have been in place for and the location within the cell. To this end the participation of RACKs seem to be
critical for appropriate targeting of PKC isoforms, although for many of the PKC isoforms the specific RACK remains unidentified. This contributes to the difficulties encountered in study of the effects of PKC isoforms. The principle effects of the different PKC isoforms on electrolyte transport and tight junction function are summarized in Figure 3.

**Phosphatidylinositol-3-kinase (PI3’K)**

Inositol containing lipids are a class of phospholipids consisting of phosphatidic acid covalently linked to an inositol ring via the 1’-OH group. If no phosphorylation of the ring has occurred, the lipid is called phosphatidylinositol (PtdIns), if the ring is phosphorylated then it is called a phosphoinositide (PI). PI3’Ks phosphorylate the 3’-OH position of the inositol ring of PtdIns and some PIs. The products of PI 3’K in mammals are PtdIns (3)P, PtdIns (3,4) P2, PtdIns (3,5) P2 and PtdIns (3,4,5) P3 or PIP3. Basal levels of PtdIns (3,4) P2, PtdIns (3,5) P2 and PtdIns (3,4,5) P3 are low, but increase dramatically after cellular stimulation. Basal PtdIns (3) P levels are much higher but remain constant. PI3’K products are not susceptible to cleavage by PLC isoenzymes that cleave inositol phospholipids into membrane bound diacylglycerol and soluble inositol phosphates. Instead they are susceptible to further phosphorylation, for example PtdIns (3) P to PtdIns (3,5) P2 or dephosphorylation by PTEN, a 3’ phosphatase that converts PtdIns(3,4)P2 to PtdIns (4)P and PIP3 to PtdIns (4,5)P2 (Payrastre et al, 2001). There are three classes of PI3’K based on their primary structure, mechanism of regulation and substrate specificity (Table 2). Other PI produced by other PIK can be
phosphorylated at the 3’ position by PI3’K, to create for example PtdIns(3,4)P2 from PtdIns(4) P, PtdIns(3,5)P2 from PtdIns(5)P, or Ptdins (3,4,5)P3 from Ptdins (4,5) P2.

The Class I PI3’K products PIP3 are typically converted to PtdIns (3,4)P2 by 5’inositol phosphatases, and are primarily located in the cytosol. Class IA PI3’K are activated by tyrosine kinases, and have 2 Src- homology-2 domains that bind phosphorylated tyrosine residues produced by tyrosine kinases (Payrastre et al. 2001, Vanhaesebroech and Waterfield, 1999). The tyrosine kinase activity can be mediated either by receptor activity or by non-receptor tyrosine kinases such as src-family kinases or JAK kinases. Class IA PI3’K (p85α-p110) is also well described in vesicles, and hence are important regulators of cell trafficking. Class IB PI3’K is activated by heterotrimeric G-protein coupled receptors and has only been found to have widespread distribution in leukocytes. Class II PI3’K C2α is identified in the trans-golgi network and the plasma membrane, but the precise in vivo function is unclear. There is increasing evidence however for their role in agonist mediated regulation of cellular functions, and activation of these kinases in LPA signaling (Payrasatre et al. 2001).

The Class III PI3’K are probably not triggered by cellular stimulation, since levels of PtdIns (3) P remain fairly constant within the cell. Nonetheless, they are critical for membrane trafficking events in response to extracellular signaling (Vanhaesebroeck and Waterfield, 1999).
The PI3’K inhibitor wortmannin binds covalently to the catalytic subunit in competition with ATP and PtdIns (4,5) P2, but not by PtdIns. The PI3’K inhibitor LY294002 is a competitive inhibitor of the ATP site. There is however, no specificity of inhibition for different classes of PI3’K with these substances. Second generation isoforms specific inhibitors are being produced (Vanhaesebroeck and Waterfield, 1999).

Recognition of PI occurs through one of two domains, the FYVE domain, or the PH domain. Proteins containing the FYVE domain favor binding PtdIns (3) P and hence interaction with Class III PI3’K. Proteins containing the PH domain favor binding either PIP3 or PtdIns(3,4)P2, products of the Class I PI3’K. PtdIns (3) P is involved in membrane trafficking and localizes with early endosomal antigen 1. EEA-1 contains a FYVE domain, and most mammalian FYVE containing proteins identified are associated with protein trafficking. The FYVE domain is less widespread than the PH domain (Vanhaesebroeck and Waterfield, 1999).

PH domains are found in many kinases (eg PKB, PDK1), phospholipases (eg PLC), nuclear exchange factors (eg Vav, ARNO), GTPase-activating factors (eg GAP1), adaptor proteins and structural proteins. GTPase-activating and exchange factors are responsible for regulation of Rho, Rac and Cdc42, which in turn are important for actin reorganization. PDK1 can activate PKA, PKG and PKC (Vanhaesebroeck and Waterfield, 1999).
The counter-regulatory mechanism that opposes PI3’K is the PtdIns 3-phosphatase, PTEN. The main substrate of PTEN is PIP3. The PtdIns 5-phosphatase SHIP1 and SHIP2 are also relevant to counter-regulation of PI3’K since they also act on PIP3 (Payrastre et al. 2001).

PIs coordinate dynamics at the interface between plasma membrane and cytoskeleton. The small GTPases, RhoA, Rac1 and Cdc42 are physically and functionally associated with PI-kinases, and can regulate the local production of PtdIns (4,5) P₂ via activation of PI(4)P 5-kinase. PtdIns (4,5)P₂ binds a variety of actin regulatory proteins, including profilin, an actin sequestering protein, it inhibits the activity of actin severing proteins, and binds capping proteins to free up the barbed end of the actin filament for further polymerization. PtdIns (4,5)P₂ may be able to control the ‘tightness’ of adhesion between the plasma membrane and underlying cytoskeleton. The mechanism of this tethering may be via α-actinin vinculin, talin or the ezrin, radixin and moesin ERM family of proteins that interact with PtdIns (4,5) P₂. PtdIns(4,5)P₂ controls the rate of turn over of α-actinin and regulates its interaction with actin filaments. N-WASP, critical for actin assembly, is activated by PtdIns (4,5) P₂. Both Rho dependent and Rho independent activation of ERM proteins require a local elevation of PtdIns (4,5) P₂ concentration (Yonemura et al. 2002). The ERM proteins cross-link integral plasma membrane proteins and actin filaments directly or indirectly, for example interacting with NHE3 through NHERF1. Rho dependent ERM activation involves the direct effector of Rho, Rho kinase phosphorylating the C-terminal threonine of ERM proteins. PtdIns (4,5)
P2 is also produced by another direct effector of Rho, and can also directly activate ERM proteins (Yonemura et al. 2002).

Oxidative stress in cells, an event that increases tight junction permeability, increases the association of PI3’K with occludin, and inhibition of PI3’K reduces the disruption of tight junction function (Sheth et al. 2003). Conversely, PI3’K is also critical for prostaglandin (PG) mediated repair of the tight junction in ischemia injured porcine ileum, and inhibition of PI3’K appears to inhibit PG mediated ZO-1 and occludin translocation back to the tight junction after ischemic injury (Little et al. 2003). PI3’K is also critical for IL-4 and IL-13 regulation of TER (Ceponis et al. 2000).

Cell migration is under tight control of the small Rho GTPases. RhoA controls assembly of stress fibers, Rac controls development of lamellipodia, and cdc42 stimulates formation of protrusions at the leading edge of cells. During cell movement, Rac and cdc42 are present at the leading edge and coordinate with RhoA at the trailing edge. High concentrations of PIP3 are identified at the front edge of migrating cells, while high concentrations of PTEN are identified at the trailing edge.

PtdIns (3,4,5)P3 can also interact with RhoA and Rac1, and binds to Vav, and GEF of Rac1. Interaction of PtdIns (3,4,5)P3 with α-actinin may also regulate restructuring of focal adhesions by disrupting interaction between integrin adhesion receptors and actin filaments.
PtdIns(4,5)P2 is required for conversion of clathrin-coated pits into clathrin-coated endosomes, and therefore conversion of this PI to PIP3 by PI3’K would be expected to inhibit clathrin-mediated endocytosis, with implications for intracellular trafficking of junctional proteins. Akt is a downstream serine/threonine kinase effector of PI3’K. NHE3 associated with lipid raft membrane microdomains, as is Akt2 and PI3’K. Akt activation is prevented by PTEN, which may also be present in the membrane microdomains. PI3’K and Akt are responsible for increasing amounts of NHE3 at the apical membrane in response to stimuli, which given the interaction with NHE3 to ezrin via NHERF, may also demonstrate the key role of PI3’K and downstream effectors in formation of plasma membrane-cytoskeletal contacts.
References


Chen ML, Pothoulakis C, LaMont JT. (2002) Protein kinase C signaling regulated ZO-1 translocation and increased paracellular flux of T84 colonocytes exposed to Clostridium difficile Toxin A. *J Biol Chem.* **277:** 4247-4254.


Figure 1: Activation and inactivation of Rho is regulated by GTP-ase activating proteins (GAP), Guanine nucleotide exchange factors (GEF) and guanosine nucleotide dissociation inhibitors (GDI). GDI bind Rho until GEF bind GTP to Rho, Rho is inactivated by GAP-mediated dephosphorylation.
Table 1: PKC isoforms, second messengers, cofactors, activators and inhibitors (DAG (diacylglycerol), Ca (Calcium), PMA (phorbol 12-myristate 13-acetate) (Way et al. 2000).

<table>
<thead>
<tr>
<th>PKC Subtype</th>
<th>Specific Isoform</th>
<th>Second messenger dependence</th>
<th>Co-factors</th>
<th>Activators</th>
<th>Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional (c)</td>
<td>α</td>
<td></td>
<td>DAG, Ca</td>
<td>Phosphatidyl-serine</td>
<td>PMA Bryostatin-1 Carbachol</td>
</tr>
<tr>
<td></td>
<td>β1, β2</td>
<td></td>
<td>DAG, Ca</td>
<td>Phosphatidyl-serine</td>
<td>Go6976 (IC50 = 2nM) Go 6850 (IC50 = 8nM) Rottlerin (IC50 = 30µM) LY 333531 (IC50 = 360nM) Go 6983 (IC50 = 7nM) Ro-31-8220 (IC50 = 5nM)</td>
</tr>
<tr>
<td></td>
<td>γ</td>
<td></td>
<td></td>
<td></td>
<td>Go 6976 (IC50 = 6nM) Go 6983 (IC50 = 7nM) Ro-31-8220 (IC50 = 24nM) LY 333531 (IC50 = 5nM)</td>
</tr>
<tr>
<td>Novel (n)</td>
<td>δ</td>
<td>DAG, not Ca</td>
<td>Phosphatidyl-serine</td>
<td>PMA Bryostatin-1 Carbachol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ε</td>
<td></td>
<td></td>
<td></td>
<td>Go6976 (IC50 = 5µM) Go6850 (IC50 = 210nM) Rottlerin (IC50 = 3-6µM) Go 6983 (IC50 = 10nM) LY 333531 (IC50 = 250nM)</td>
</tr>
<tr>
<td></td>
<td>η</td>
<td></td>
<td></td>
<td>Rottlerin (IC50= 82µM) LY333531 (IC50=52nM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>μ (≡PKD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>θ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atypical (a)</td>
<td>ζ</td>
<td>Not DAG or Ca</td>
<td>Phosphatidyl-serine</td>
<td>Not PMA or bryostatin-1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>τ/λ</td>
<td></td>
<td></td>
<td>Go 6983 (IC50= 60nM) Go 6850 (IC50 = 5.8µM)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2: Schematic representing epithelial chloride secretion.
Figure 3: Schematic summarizing major effects of different PKC isoforms on tight junction and secretory function. Isoforms outlined in red are generally inhibitory at the locations indicated, isoforms outlined in green generally activate at the locations indicated, and those outlined in orange either activate or inhibit, depending on the cellular conditions.
Table 2: Classes, composition, substrates and products of different PI3’K isoforms.

<table>
<thead>
<tr>
<th>Class</th>
<th>Catalytic Subunit</th>
<th>Adaptor/Regulatory Subunit</th>
<th>Lipid substrates $\textit{In vivo}$</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA</td>
<td>p110 $\alpha\beta\delta$</td>
<td>P85$\alpha$, p85$\beta$, p85$\gamma$</td>
<td>PtdIns (4,5)P$_2$</td>
<td>PIP3</td>
</tr>
<tr>
<td>IB</td>
<td>p110$\gamma$</td>
<td>P101</td>
<td>PtdIns (4,5)P$_2$</td>
<td>PIIP3</td>
</tr>
<tr>
<td>II</td>
<td>PI3-K C2$\alpha\beta\delta$</td>
<td>?</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>Vps34p analogues</td>
<td>P150</td>
<td>PtdIns</td>
<td>PtdIns (3)P</td>
</tr>
</tbody>
</table>
Chapter 2

Phosphatidylinositol-3-kinase (PI3'K) signaling is required for prostaglandin-induced mucosal recovery in ischemia-injured porcine ileum

Dianne Little,¹ Rebecca A. Dean,¹ Karen M. Young,¹ Shaun A. McKane,² Linda A. Martin,² Samuel L. Jones,¹ Anthony T. Blikslager¹

Departments of ¹Clinical Sciences, and ²Molecular Biomedical Sciences, North Carolina State University, Raleigh, North Carolina

Short Title: Prostaglandin-induced recovery of ischemia-injured ileum

Funding and technical support: NIH Grant DK53284 (to ATB) and USDA National Research Initiative Grant 0102490 (to ATB and SLJ).

Abbreviations used in this paper: PI3’K, Phosphatidylinositol-3-kinase; TER, transepithelial electrical resistance; Isc, short circuit current; J_m, mucosal-to-serosal flux; J_s, serosal-to-mucosal flux.
**Introduction**

Restoration of the intestinal mucosal barrier following a variety of injurious or inflammatory events is a critical component of innate mucosal defense (Podolsky 1999). In previous studies, we have begun to elucidate the pathways by which the prostanoids PGE$_2$ and PGI$_2$ stimulate recovery of barrier function in porcine ischemia-injured ileal mucosa. In particular, we have noted that this recovery process appears to be related to events localized to the paracellular space rather than reparative events such as epithelial restitution and villous contraction (Blikslager *et al.* 1997, Blikslager *et al.* 1999, Blikslager *et al.* 2000). However, studies by Zushi (1996) have shown that PGE$_2$ is permissive for growth factor-stimulated restitution and PGE$_2$ stimulates contraction of uninjured villi and crypts (Erickson 1990), suggesting the reparative actions of PGs are multiple and complex. In porcine ileal mucosa subjected to 45-minutes ischemia, villous contraction and epithelial restitution are near complete within 60-minutes of injury, and yet PGs are able to stimulate continued elevations in TER after 60-minutes. These elevations in TER are correlated with decreased transmucosal flux of the paracellular probes mannitol and inulin, and electron-microscopic evidence of closure of paracellular spaces in restituted epithelium (Blikslager *et al.* 1999, Blikslager *et al.* 2000). Furthermore, PG-induced elevations in TER are inhibited by cytochalasin D (Blikslager *et al.* 1997), an agent that initiates cytoskeletal contraction and opening of tight junctions at the appropriate dosages (Madara *et al.* 1987).

The mechanisms by which PGs stimulate closure of paracellular spaces are not fully characterized, although we know that sharp elevations in Cl$^-$ secretion precede recovery, and
inhibition of Cl⁻ secretion with the loop diuretic bumetanide attenuates mucosal recovery (Blikslager et al. 1999). The role of Cl⁻ secretion in recovery of paracellular resistance is unclear, although it is conceivable that this event results in a transmucosal osmotic gradient. Indeed, mucosal osmotic loads have been shown to stimulate elevations in TER in normal guinea pig ileum (Madara 1983), and recovery of TER in ischemia-injured porcine ileal mucosa (Blikslager et al. 1999). We have speculated that initial repair of tight junctions would have to precede their subsequent closure and recovery of TER (Blikslager et al. 2000).

Prostaglandin signaling mechanisms that might result in tight junction repair include their second messengers cAMP and Ca²⁺ (Blikslager et al. 1997), both of which have been shown to alter tight junction structure in Necturus gallbladder (Duffey et al. 1981, Palant et al. 1983). Additional signaling intermediates that we have investigated are tyrosine kinases, and protein kinase C (Blikslager et al. 2000). Although genistein augmented prostaglandin-induced mucosal recovery, this did not appear to relate to its ability to inhibit tyrosine kinases, and inhibition of protein kinase C had no effect on prostaglandin-stimulated mucosal recovery (Blikslager et al. 2000). However, recent evidence suggests that PI3’K is intimately involved in regulation of tight junction assembly (Woo et al. 1999), and preferentially binds to specific regions of the transmembrane protein occludin via its p85 regulatory subunit (Nusrat et al. 2000). Therefore, in the present study, we sought to provide further evidence for a selective action of prostaglandins on recovery of paracellular resistance, and to determine if PI3’K plays a role in this reparative process. Our data show that inhibition of PI3’K completely inhibits the action of PGs, which is correlated with inhibition of the ability
of PGs to restore localization of the tight junction integral membrane protein occludin and the cytoplasmic plaque protein ZO-1 to inter-epithelial junctions.

Materials and Methods

Experimental animal surgeries

All studies were approved by the North Carolina State University Institutional Animal Care and Use Committee. Six to eight-week-old Yorkshire crossbred pigs of either sex were housed singularly, and maintained on a commercial pelleted feed. Pigs were held off feed for 24 hours prior to experimental surgery. General anesthesia was induced with xylazine (1.5 mg/kg, IM), ketamine (11 mg/kg, IM) and pentobarbital (15mg/kg, IV) and was maintained with intermittent infusion of pentobarbital (6-8mg/kg/hr). Pigs were placed on a heating pad and ventilated with 100% O$_2$ via a tracheotomy using a time-cycled ventilator. The jugular vein and carotid artery were cannulated, and blood gas analysis was performed to confirm normal pH, and partial pressures of CO$_2$ and O$_2$. Lactated Ringer’s solution was administered intravenously at a maintenance rate of 15ml/kg/hr. Blood pressure was continuously monitored via a transducer connected to the carotid artery. The ileum was approached via a ventral midline incision. Ileal segments were delineated by ligating the intestinal lumen at 10-cm intervals. Loops were randomly designated as control or ischemic loops. The latter were subjected to ischemia by clamping the local mesenteric blood supply for 45-minutes.
Ussing chamber studies

Following the ischemic period, the mucosa was stripped from the seromuscular layer in oxygenated (95% O₂/ 5% CO₂) Ringer’s solution, and mounted in 3.14 cm² aperture Ussing chambers, as described in a previous study (Argenzio and Liacos 1990). Tissues were bathed on the serosal and mucosal sides with 10ml Ringer’s solution. The serosal bathing solution contained 10mM glucose, and was osmotically balanced on the mucosal side with 10mM mannitol. Bathing solutions were oxygenated (95% O₂/5% CO₂), 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-minutes for 180-minutes.

Experimental treatments

Tissues were bathed in Ringer’s containing 5µM indomethacin to prevent PG production while stripping mucosa from the seromuscular tissues, and indomethacin was added to the serosal and mucosal bathing solutions in the same concentration prior to mounting tissues on Ussing chambers. Other treatments that were added to the serosal and mucosal bating solutions prior to baseline electrical measurements were the PI3’K inhibitors wortmannin (10nM) and LY294002 (10µM). Baseline electrical readings were taken for 30-minutes, after
which 1µM 16,16-dimethyl-PGE\(_2\) (Sigma Chemical Co., MO) and 1µM carbacyclin (Sigma Chemical Co., MO), the stable analog of PGI\(_2\), were added to the serosal bathing solution. In studies assessing the role of osmotic gradients, 100-300mOsm urea was added to either the mucosal or serosal side of tissues. In studies in which hydrostatic pressures were applied, the volume of fluid was incrementally increased on the serosal side of tissues by 1-3ml.

*Isotopic mannitol and Na\(^+\) flux studies*

All fluxes were conducted under short-circuit conditions (tissues clamped to 0mV). Dual transmucosal mannitol and Na\(^+\) fluxes were performed on tissues paired according to their initial conductance readings (within 25% of each other). \(^3\)H-mannitol (0.2 µCi/ml diluted in 10mM mannitol) or \(^14\)C-inulin were placed on the mucosal side of tissues and 0.3µCi/ml \(^22\)Na was placed on the serosal side of tissues following an initial 30-minute equilibration period. One 60-minute flux was subsequently conducted from 60-120-minutes of the experimental recovery period by taking samples opposite from the side opposite to that of isotope addition, and counted for \(^3\)H or \(^22\)Na in a scintillation counter. Mucosal-to-serosal fluxes (J\(_{ms}\)) of mannitol or inulin and serosal-to-mucosal fluxes (J\(_{sm}\)) of Na\(^+\) were calculated using standard equations (Arrenziolo and Liacos 1990, Argenzio et al. 1993).

*Electron and light microscopy*

Tissues were taken at 0, 30, 60, 120, and 180-minutes for routine histologic evaluation. Tissues were sectioned (5 µm) and stained with hematoxylin and eosin. For each tissue, 3 sections were evaluated. Four well-oriented villi were identified in each section. 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, and 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 (Argenzio et al. 1993). 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 villous surface area was used as an index of epithelial restitution.

In experiments designed to assess epithelial ultrastructure under the influence of PGs, tissues were removed from Ussing chambers after 120-minutes (peak TER) during 3 separate experiments. Tissues were placed in Trump’s 4F:1G fixative, and prepared for transmission electron microscopy using standard techniques (Dykstra 1994). For each tissue evaluated, 5 well-oriented interepithelial junctions were evaluated. A calibrated grid was placed over electronmicrographs extending from the apical most aspect of the interepithelial space to 3µm deep to the apical membrane, and 1.5µm from either side of the apical interepithelial space so that the entire grid encompassed 9µm². The number of squares that were occupied...
by paracellular space within this 9µm² grid was used to calculate the area of the paracellular space.

**Epithelial isolation**

Tissues were rinsed with 30ml of cold CO₂ saturated PBS, and subsequently dropped into a tube containing CO₂ saturated citrate phosphate buffer (96 mM NaCl, 1.5mM KCl, 27.0mM Na Citrate, 5.6mM KH₂PO₄, 8.0mM Na₂HPO₄). The tube was capped immediately, and incubated at 37°C in a water bath for 20 minutes. The tissue was then transferred to a tube containing CO₂ EDTA buffer (137mM NaCl, 2.7mM KCl, 1.5mM KH₂PO₄, 8.0mM Na₂HPO₄, 1.5mM Tetrasodium EDTA, 2.5 mM Glucose) and incubated at 37°C in a water bath for 30 minutes. Tissues were vortexed, after which a histologic sample was submitted to check for the degree of epithelial sloughing. Tissues were subsequently centrifuged at 2000rpm for 10 minutes, and the pellet solubilized in EDTA buffer in preparation for Western blotting.

**Gel electrophoresis and Western blotting**

Isolated epithelium from control and ischemia-injured mucosa treated with indomethacin (5µM), indomethacin (5µM) and PGs (1µM) or indomethacin (5µM), PGs (1µM) and wortmannin (10nM) and recovered for 120-minutes in oxygenated Ringer’s was snap frozen and stored at -70°C prior to SDS-PAGE. Tissue aliquots were thawed at 4°C, and added to 3 ml chilled lysis buffer, including protease inhibitors (0.5mM Pefabloc, 0.1 mM 4-nitrophenyl phosphate, 0.04mM β-glycerophosphate, 0.1 mM Na₃VO₄, 40µg/ml bestatin, 2µg/ml
aprotinin, 0.54 µg/ml leupeptin, and 0.7 µg/ml pepstatin A) at 4°C. This mixture was homogenized on ice, then centrifuged 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. Lysates were loaded on a 10% SDS-polyacrylamide gel and electrophoresis carried out according to standard protocols. Proteins were transferred to a nitrocellulose membrane (Hybond ECL, Amersham Life Science, Birmingham, UK) using an electro-blotting mini-transfer apparatus. Membranes were blocked at room temperature for 60-minutes in Tris-buffered saline plus 0.05% Tween-20 and 5% dry powdered milk. Membranes were washed, then incubated for 60-minutes in primary antibody. After washing, the membranes were incubated for 45-minutes with horseradish peroxidase conjugated secondary antibody. After additional washing, the membranes were developed for visualization of protein by addition of enhanced chemiluminescence reagent (Amersham, Princeton NJ). Densitometry was performed using appropriate software (IP gel, Scanalytics, Fairfax VA).

**Immunofluorescence microscopy**

Tissues were fixed in 10% neutral-buffered formalin for 24-hours, transferred to 70% ethanol, routinely processed for paraffin embedding and cut into 5µM sections. Slides were subsequently deparaffinized and rehydrated. Epitope retrieval was done by boiling the specimens in citrate buffer (pH 6.0) for 10 minutes, then allowing specimens to cool for 25 minutes at room temperature. Sections were blocked with 2% BSA, and washed with bovine...
lacto transfer technique optimizer (BLOTTO) and PBS, after which they were incubated in primary rabbit polyclonal anti-occludin, primary rabbit polyclonal anti-ZO-1, or an isotype control for rabbit primary antibody (negative control) for one hour on ice. Sections were then incubated with Goat anti-rabbit IgG Cy3 conjugate for 30 minutes in the dark. Sections were mounted and well-oriented villi examined with an immunofluorescence microscope.

Data analysis

Data were reported as mean ± SE. All data were analyzed using an ANOVA for repeated measures except where the peak response was analyzed using a standard one way ANOVA or paired t-test (Sigmastat, Jandel Scientific, San Rafael, CA). A Tukey’s test was used to determine differences between treatments following ANOVA. Flux data was subjected to linear regression analysis, and the correlation coefficient (R) assessed for significance. P < 0.05 was considered significant for all analyses.

Results

Application of 1µM 16,16-dimethyl PGE$_2$ and 1µM carbacyclin (a stable analog of PGI$_2$) to mucosal sheets of porcine ileum injured by 45-minutes of ischemia and bathed in 5µM indomethacin resulted in recovery of control levels of TER within 30-minutes, whereas ischemia-injured tissues exposed to indomethacin alone showed minimal elevations of TER over a 180-minute recovery period (Figure 1A). As we have shown in previous reports, this PG-induced recovery was preceded by a sharp elevation in Isc (Figure 1B) attributable to secretion of Cl$^-$ (Blikslager et al. 1999). As in previous studies (Blikslager et al. 2000), there
was no difference in the histologic appearance of repairing tissues treated with indomethacin compared to those additionally treated with PGs (Figure 2), which was confirmed by showing no significant difference in the degree of epithelial restitution (Table 1). In fact, restitution was near complete within 60-minutes, suggesting that the peak effects of PGs between 90-120-minutes were related to events localized to the paracellular space.

To further explore the possibility that PG-induced changes in TER were paracellular in nature, we measured mucosal-to-serosal fluxes of the paracellular probes $^3$H-mannitol and $^{14}$C-inulin as well as serosal-to-mucosal fluxes of $^{22}$Na$^+$ between 60 and 120-minutes of the recovery period (when PG-treated tissues reached maximum TER values). Flux of these probes was significantly greater in ischemia-injured tissues treated with indomethacin alone compared to tissues treated additionally with PGs (Figure 3). We then assessed the correlation between the flux of mannitol or inulin and that of Na$^+$ as a method of assessing the contribution of changes in paracellular permeability (accounted for by mannitol or inulin flux) to changes in TER (accounted for by serosal-to-mucosal Na$^+$ flux), as previously described (Madara et al. 1988). We first confirmed that $J_{\text{na sm}}^\text{na}$ closely correlated with changes in TER in tissues treated with indomethacin or indomethacin/PGs ($R=0.76, P<0.001$, data not shown). We subsequently documented a significant and linear correlation between fluxes of the paracellular probes and $J_{\text{na sm}}^\text{na}$ (Figure 4), indicating that changes in resistance were indeed reflective of changes in paracellular permeability.
Although the experiments thus far indicated an action of PGs on the paracellular space, we wanted more direct evidence of the involvement of the paracellular structures in the recovery response. Therefore, we performed a series of experiments in which we added increasing levels of serosal hydrostatic pressure by raising the fluid level of the serosal reservoir. We postulated that this would dilate paracellular spaces and apical tight junctions, thereby nullifying the effects of PGs. Accordingly, there was a pressure-dependent decrease in the PG-induced recovery of TER, with 6-cm serosal pressure nullifying the effects of PGs on injured tissues (Figure 5). This action was not attributable to disruption of Cl⁻ secretion, since there was no significant reduction of Isc by 6-cm serosal pressure. Tissues taken during peak TER levels in response to PGs showed ultrastructural evidence of closely apposed tight junctions compared to tissues treated with indomethacin alone. Furthermore, tissues subjected to 6cm of serosal pressure in the presence of PGs also showed dilatation of paracellular structures (Figure 6). These observations were confirmed morphometrically by showing pressure-dependent increases in the area of the paracellular space (Figure 7). There was no effect of hydrostatic pressure on normal tissues (data not shown); suggesting hydrostatic pressure selectively affected tissues in the process of recovering paracellular resistance.

In further experiments, we attempted to elucidate some of the mechanisms involved in PG-induced recovery of paracellular resistance. In previous studies, we have suggested that increases in Cl⁻ secretion that precede recovery of TER may result in development of an osmotic gradient across the mucosa (Blikslager et al. 1999, Blikslager et al. 2000). To test
this hypothesis, we applied increasing doses of urea on the mucosal surface of ischemia-
injured tissues treated with indomethacin, and compared the effects of these treatments with
that of the PGs. Accordingly, we noted dose-dependent increases in recovery of TER with
mucosal application of urea that peaked with application of 200mOsm (Figure 8).
Application of other osmotic agents to the mucosal surface of tissues, including mannitol
(300mOsm) and lactulose (300mOsm), resulted in similar increases in TER in ischemia-
injured mucosa (peak TER in response to mannitol, 66±4Ω·cm², n=6; peak TER in response
to lactulose, 65±2Ω·cm², n=3). To demonstrate the importance of the direction of the osmotic
gradient, we applied 300mOsm urea to the serosal surface of ischemia-injured tissues, and
saw a reduction rather than an increase in recovery of TER. We next reasoned that if PGs
were setting up a mucosal-to-serosal osmotic gradient, the effect of the PGs should be
reversed with serosal application of urea. In support of this premise, application of 300mOsm
urea to the serosal surface of recovering tissues fully inhibited the action of PGs on injured
tissues (Figure 8).

In previous studies, we have postulated that tight junction re-assembly would be required to
initiate recovery of TER (Blikslager et al. 2000). Because of studies implicating PI3’K in
tight junction assembly (Woo et al. 1999), we were particularly interested in this signaling
pathway. Application of the PI3’K inhibitor wortmannin (10nM) completely inhibited PG-
induced recovery, but had no effect on Isc in PG-treated tissues (Figure 9). To rule out an
effect of wortmannin on restitution, we calculated the percentage of denuded mucosa during
in vitro recovery, as in our initial experiments. Following a 60-minute recovery period, there
was no significant effect of wortmannin on percent denuded mucosa (1.9±1.4%) compared to other treatment groups (Table 1), suggesting wortmannin inhibited paracellular effects of PG addition. However, wortmannin appeared to reduce the small recovery response of ischemia-injured tissues treated with indomethacin alone, suggesting the possibility of non-specific toxic effects of wortmannin. Therefore, we also assessed the effects of the alternative PI3′K inhibitor LY294002 (10µM). This agent fully inhibited recovery of TER in PG-treated tissues. However, LY294002 did not fully inhibit the PG-stimulated elevations in Isc, which remained significantly elevated when compared to tissues treated with indomethacin alone (Figure 10). LY294002 appeared to have no effect on TER or Isc measurements when applied to ischemia-injured tissues treated with indomethacin in the absence of PGs.

Since we have postulated that PG-induced Cl− secretion sets up an osmotic gradient that is in turn responsible for at least part of the recovery of paracellular resistance, we wanted to determine if the PI3′K inhibitor LY294002 would also inhibit urea-stimulated recovery. Therefore, tissues were treated with 200mOsm urea on their mucosal surface in the presence or absence of LY294002 (10µM). The PI3′K inhibitor LY294002 inhibited the effect of urea (Figure 11), suggesting PI3′K signaling is required for osmotic load-induced recovery of TER similar to that of PG-induced recovery of TER. Similar results were obtained in tissues pre-treated with wortmannin (data not shown).

In additional experiments assessing PI3′K-mediated events, we sought to further define the role of PGs and PI3′K inhibitors on select components of the tight junction, since it is this
structure that is largely responsible for regulating paracellular permeability (Madara 1990). Therefore, we assessed the tissue expression of the tight junction transmembrane proteins occludin and claudin-5 following 120-minutes of recovery in the presence of PGs and wortmannin. We used a technique to isolate epithelial cells from remaining mucosal elements in order to be sure that we were not detecting tight junction proteins from other tissues such as endothelium. Microscopic studies confirmed complete epithelial separation from mucosal villi following the isolation procedure (data not shown). Indomethacin appeared to reduce the expression of claudin-5 in ischemia-injured mucosal epithelium, but there was no apparent effect of any of our treatments on occludin expression (Figure 12). However, in further studies using immunofluorescence microscopy, we noted differences in the distribution of occludin in the various treatment groups (Figure 13). In particular, we noted inter-epithelial localization of occludin labeling in control epithelium that was disrupted in ischemia-injured tissue bathed in indomethacin (5µM) for 120-minutes. Treatment with PGs (1µM) appeared to restore the normal inter-epithelial junctional distribution of occludin, whereas pre-treatment of tissues with wortmannin (10nM) inhibited the ability of PGs to restore occludin distribution. In order to seek further evidence of tight junction structural restoration in the presence of PGs, we also performed immunofluorescence experiments to assess the localization of the tight junction cytoplasmic plaque protein ZO-1. These experiments revealed highly selective localization of ZO-1 to the tight junction in control tissues, and ischemia-injured tissues exposed to PGs (1µM). In contrast, ischemia-injured tissues recovered in the presence of indomethacin (5µM) alone or with wortmannin (10nM) had evidence of diffuse staining in the apical region of recovering epithelial cells (Figure 14).
Discussion

Mechanisms believed to be critical for recovery of injured epithelium include restitution (Moore et al. 1989, Nusrat et al. 1992) and, in the case of small intestinal mucosa, villous contraction (Moore et al. 1989). Restitution is a broad term that denotes recovery of an intact monolayer of epithelium across a previously denuded region of the mucosa (Podolsky 1999). Thus, restitution may be broken down into epithelial migratory events, and tight junction resealing events. Prostaglandins have not been extensively linked to villous contraction or epithelial migration, although there is evidence that PGE₂ stimulates contraction of villi in normal mucosa (Erickson 1990), and the cyclooxygenase inhibitor piroxicam suppresses epithelial migration stimulated by growth factors in cultured intestinal epithelial cells (Zushi 1996). However, we have not found any evidence for an effect of PGs on either villous contraction or epithelial migration in ischemia-injured porcine ileal mucosa. For example, tissues exposed to 45-minutes of ischemia have histologic evidence of a complete epithelial monolayer after 60-minutes of in vitro recovery in tissues regardless of whether they are treated with indomethacin alone or indomethacin and exogenous PGs (Figure 2).

Nonetheless, PGs stimulate significant elevations in TER and reductions in permeability to mannitol and inulin, leading us to focus on potential effects of PGs on paracellular structures. The present studies provide further evidence for an effect of PGs on the paracellular space. For example, serosal-to-mucosal Na⁺ fluxes (which reflect changes in TER) significantly correlated with mucosal-to-serosal fluxes of the paracellular probes inulin and mannitol. Furthermore, quantitation of the dimensions of the junctional region of the paracellular space revealed significant reductions in the area of this space in response to PGs, whereas serosal
hydrostatic pressure significantly increased the area of the paracellular space and inhibited the actions of PGs on recovery of TER.

Since tight junctions largely regulate paracellular permeability, it is likely that at least a component of the action of PGs is directed at these structures. Immunofluorescence of occludin and ZO-1 would tend to support this conclusion, since localization of these tight junction proteins to the region of the inter-epithelial junctions was associated with peak TER in response to PGs. However, it is also possible that PGs have an effect on the sub-junctional paracellular space, the collapse of which might be responsible for a component of the recovery of TER. The importance of the proximity of epithelial lateral membranes has previously been shown to influence measurements of TER (Kottra and Fromter 1993, Kottra et al. 1993), and the experiments with serosal pressure supports the idea that dilating the paracellular space reduces the ability of PGs to stimulated recovery of TER. However, electronmicrographs also showed evidence of dilation of the tight junction in response to serosal pressure, making it difficult to separate the effects of this maneuver on the paracellular space and the tight junction. Similarly, ischemia-injured tissues treated with indomethacin alone had dilated tight junctions and paracellular spaces, whereas those treated with PGs had closely apposed tight junctions and paracellular spaces. However, it is likely that tight re-sealing precedes collapse of the subjacent paracellular space because the continued presence of a dilated tight junction would allow extracellular fluid to enter the paracellular space.
Mechanisms of tight junction re-sealing following ischemia have not been fully characterized. Firstly, it is likely that tight junctions have to re-assemble following ischemic injury, since ischemia or associated ATP depletion disrupts tight junction integrity (Mandel et al. 1993, Molitoris et al. 1989). Re-assembly of tight junctions following events such as ATP depletion involves localization of integral membrane proteins such as occludin to the apical-most aspect of the lateral epithelial membrane, along with colocalization of cytoplasmic proteins such as ZO-1 (Ye et al. 1999). Similarly, studies utilizing a calcium switch (chelation and subsequent repletion of calcium) to disrupt and allow recovery of tight junctions documented the critical role of integral membrane proteins in orchestrating re-assembly of tight junctions (Nusrat et al. 1995). Although we do not know if these same mechanisms are responsible for PG-stimulated recovery of TER, we do have evidence on immunofluorescence that PGs restore the distribution of the tight junction integral membrane protein occludin and the cytoplasmic plaque protein ZO-1 to the region of the inter-epithelial junction. However, there was no difference in the expression of occludin in response to PG treatment, suggesting that PGs stimulate movement of pre-existing occludin dispersed throughout the cell during ischemic injury to the inter-epithelial junction during recovery.

In the present study, we were able to use PI3’K inhibitors to functionally separate changes in Isc and TER, both of which are stimulated by PGs. We know from previous studies that inhibition of Isc and the associated secretion of Cl⁻ largely blocks the action of PGs on recovery of TER (Blikslager et al. 1999). However, it now appears that PI3’K-mediated events are critical for recovery of TER despite the continued presence of elevations in Isc.
Inhibitors of PI3’K also blocked recovery of TER in response to mucosal osmotic loads of urea. Taken together, this data suggests that PI3’K-mediated events are downstream of mechanisms resulting in a mucosal-to-serosal osmotic gradients, including mucosal urea and PG-induced Cl\(^{-}\) secretion (proposed model shown in Figure 15). However, as an alternate possibility, it is also conceivable that PGs require both elevations in Isc and intact PI3’K signaling in order to stimulate recovery of TER. As far as the specific mechanisms involved in PI3’K-sensitive recovery of TER, this will have to await further study. However, previous studies demonstrating preferential binding of the p38 regulatory domain of PI3’K to occludin (Nusrat et al. 2000) and a role for PI3’K in junctional actin re-arrangement (Nybom and Magnusson 1996) suggest potential important functions of this enzyme in tight junction re-sealing.
References


Blikslager AT, Roberts MC, Rhoads JM, Argenzio RA. (1997) Prostaglandins I$_2$ and E$_2$ have a synergistic role in rescuing epithelial barrier function in porcine ileum. *J Clin Invest.* **100:** 1928-1933.


Madara JL. (1983) Increases in guinea pig small intestinal transepithelial resistance induced by osmotic loads are accompanied by rapid alterations in absorptive-cell tight-junction structure. *J Cell Biol.* **97**: 125-136.


Table 1. Morphometric assessment of epithelial restitution (measured as percentage of the villous surface area that remained denuded) in ischemia-injured porcine ileal mucosa treated with indomethacin (5µM), and carbacyclin and PGE₂ (PGs, 1µM). *P<0.05 vs. Ischemia at 0 minutes and ischemia/ indomethacin at 30-minutes.

<table>
<thead>
<tr>
<th>Treatment (n=6)</th>
<th>Recovery time (minutes)</th>
<th>Villous surface area denuded (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ischemia</td>
<td>0</td>
<td>37.2 ± 5.4</td>
</tr>
<tr>
<td>Ischemia/ indomethacin</td>
<td>30</td>
<td>10.1 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>5.6 ± 2.4*</td>
</tr>
<tr>
<td>Ischemia/ indomethacin/ PGs</td>
<td>60</td>
<td>3.4 ± 1.1*</td>
</tr>
</tbody>
</table>
Figure 1. Electrical responses of ischemia-injured tissues treated with indomethacin (indo, 5μM), 16,16-dimethyl prostaglandin E₂ and carbacyclin (PGs, 1μM). A: Serosal addition of PGs to ischemia-injured tissues after an initial 30-minute equilibration period resulted in rapid recovery of control levels of transepithelial electrical resistance (TER), whereas tissues treated with indomethacin alone had little evidence of recovery. B: Elevations in TER were preceded by significant elevations in Isc, which is associated with Cl⁻ secretion in this tissue. Plotted values represent mean ± SE, n=8. The significance of the elevations in TER and Isc in the presence of PGs was determined using 2-way ANOVA on repeated measures (P<0.05).
**Figure 2.** Histologic appearance of ischemia-injured porcine ileal mucosa. **A:** Ischemia for 45 minutes resulted in lifting and sloughing of epithelium from the tips of villi. **B:** After a 60-minute recovery period in a Ussing chamber in the presence of indomethacin (5µM), villi have contracted and epithelial restitution is near complete. **C:** Tissues treated with indomethacin and PGs (added after a 30-minute equilibration period, and recovered for an additional 30-minutes) have a similar histologic appearance as tissues treated with indomethacin alone. 1cm bar = 100µm.
Figure 3. Evaluation of the effects of indomethacin and prostaglandins on serosal-to-mucosal fluxes of $^{22}$Na, and mucosal-to-serosal fluxes of $^{3}$H-mannitol and $^{14}$C-inulin. Fluxes were commenced 30-minutes following the addition of PGs, and were conducted over a 1-hour period. A: Tissues in the presence of indomethacin (5µM) and PGs (1µM) had significantly reduced $J_{sm}$ $^{22}$Na over a 1-hour time flux period compared to tissues treated with indomethacin alone. Similar results were obtained for mucosal-to-serosal fluxes of the paracellular probes $^{3}$H-mannitol (B) and $^{14}$C-inulin (C). Plotted values represent mean ± SE, n=8. *P < 0.05 vs. indomethacin-treated tissues.
Figure 4. Correlation between $J_{sm}^{22}\text{Na}$ and $J_{ms}^{3}\text{H}-\text{mannitol}$ or $J_{ms}^{14}\text{C}-\text{inulin}$ in ischemia-injured tissues treated with indomethacin (5µM) and PGs (1µM) or indomethacin alone. There was a significant correlation between $J_{sm}^{22}\text{Na}$ and $J_{ms}^{\text{mannitol}}$ (A) or $J_{ms}^{\text{inulin}}$ (B), suggesting that changes in transepithelial electrical resistance (which correlate closely with $J_{sm}^{22}\text{Na}$) were related to changes in paracellular permeability (as indicated by changes in $J_{ms}$ of the paracellular probes mannitol and inulin). The correlation coefficient (R) and its significance (P values) are indicated adjacent to linear regression plots.
A

\[ J_{ms}^{3}\text{H-mannitol} \]

Ischemia/ indo
Ischemia/ indo/ PGs

\[ R=0.61, P<0.001 \]

B

\[ J_{ms}^{14}\text{C-inulin} \]

\[ R=54, P<0.01 \]

\[ J_{sm}^{22}\text{Na (µEq/cm}^2\text{.h)} \]
**Figure 5.** Effects of serosal hydrostatic pressure on recovery of transepithelial resistance.  
A: The recovery response of ischemia-injured tissues treated with indomethacin (5µM) and PGs (1µM) was marginally reduced by 2-4cm of serosal pressure, whereas 6cm of hydrostatic pressure fully and significantly inhibited recovery of transepithelial electrical resistance.  
B: Changes in short circuit current (Isc) in response to serosal pressure did not appear to be correlated with changes in TER. In particular, 6cm of water caused a small increase in Isc, but fully inhibited TER, suggesting that the effects of serosal pressure related to mechanical effects on the paracellular space, rather than alterations in Cl− secretion. Plotted values represent mean ± SE, n=8. The significant reduction in TER in the presence of PGs/ 6cm water compared to tissues in the presence of PGs alone was determined using 2-way ANOVA on repeated measures (P<0.05).
Resistance ($\Omega \cdot \text{cm}^2$)

Ischemia/indo

Ischemia/indo/PGs

Ischemia/indo/PGs/2cm pressure

Ischemia/indo/PGs/4cm pressure

Ischemia/indo/PGs/6cm pressure

Add PGs

Isc ($\mu\text{A}/\text{cm}^2$)

Time (minutes)
Figure 6. Electronmicrographs of tissues exposed to indomethacin, prostaglandins, and serosal pressure. **A**: Ischemia-injured tissues after a 120-minute *in vitro* recovery period in the presence of indomethacin (5µM) have dilated tight junctions (arrows) and paracellular spaces. **B**: Tissues additionally treated with PGs (1mM) have closely apposed tight junctions (arrows). **C**: Application of 6cm serosal hydrostatic pressure to tissues treated with both indomethacin and PGs results in dilatation of tight junctions (arrows) and paracellular spaces. 1cm bar = 2µm **D**: Increased magnification of the inter-epithelial junctional region of ischemia-injured tissues treated with indomethacin for 120-minutes. Note dilatation of the tight junction (arrow). **E**: The same high power magnification of the inter-epithelial junctional region of ischemia-injured tissues treated with indomethacin and PGs revealed closely apposed tight junctions (arrow), whereas tissues exposed to 6cm serosal hydrostatic pressure (**F**) had dilated tight junctions (arrow). 1cm bar = 1µm
**Figure 7.** Area of the paracellular space in the region of the tight junction based on electron-microscopic images. The area of the paracellular space was dramatically reduced in tissues treated with indomethacin (5µM) and PGs (1µM) compared to tissues treated with indomethacin alone. Application of 2-4cm serosal pressure had no significant effect on area of the paracellular space compared to tissues treated with indomethacin and PGs, but 6cm hydrostatic pressure caused a significant elevation in the area of the paracellular space. Plotted values represent mean ± SE, n=8. *P<0.05 vs. ischemia/indomethacin tissues. †P<0.05 vs. all other treatment groups, as determined by 1-way ANOVA and a post-hoc Tukey’s test.
Figure 8. Electrical responses of tissues subjected to osmotic loads of urea. Dose-dependent increases in TER were noted in response to 100-200mOsm urea on the mucosal surface of tissues, whereas 300mOsm urea appeared to have no further effect. Conversely, serosal application of 300mOsm reduced TER below levels of tissues treated with indomethacin alone and fully inhibited recovery of tissues in response to PGs, suggesting the orientation of osmotic gradients in recovering tissue is critical. Plotted values represent mean ± SE, n=8. The significant increases in TER in the presence of 200-300mOsm mucosal urea, and the significant inhibitory effect of 300mOsm serosal urea was determined using 2-way ANOVA on repeated measures (P<0.05).
Figure 9. Electrical responses of tissues to inhibition of phosphatidylinositol-3-kinase (PI3’K). A: Pre-treatment with the PI3’K inhibitor wortmannin (10nM) fully inhibited recovery of TER in tissues treated with indomethacin (5µM) and PGs (1µM). B: Short circuit current (Isc) in tissues treated with indomethacin and PGs was no different than Isc levels in tissues additionally treated with wortmannin, suggesting inhibition of TER did not relate to blockade of Isc. Significant inhibitory effect of wortmannin on tissues treated with indomethacin and PGs was determined using 2-way ANOVA on repeated measures (P<0.05).
Resistance ($\Omega \cdot \text{cm}^2$)

Ischemia/indo
Ischemia/indo/wortmannin
Ischemia/indo/PGs
Ischemia/indo/wortmannin/PGs

Add PGs

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Isc ($\mu$A/cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td>90</td>
<td>30</td>
</tr>
<tr>
<td>120</td>
<td>40</td>
</tr>
<tr>
<td>150</td>
<td>50</td>
</tr>
<tr>
<td>180</td>
<td>60</td>
</tr>
</tbody>
</table>

A

B
Figure 10. Electrical responses of tissues to inhibition of phosphatidylinositol-3-kinase (PI3’K) with LY294002. A: Tissues pre-treated with the alternative PI3’K inhibitor LY294002 (10µM) had no evidence of recovery of TER in response to treatment with PGs, whereas LY294002 had no effect on tissues treated solely with indomethacin. B: Short circuit current (Isc) in tissues pre-treated with indomethacin and PGs was partially inhibited by LY294002. However, in previous studies,(Blikslager et al. 1999) we have shown that complete inhibition of Isc is required to inhibit recovery of TER, suggesting inhibition of recovery of TER by LY294002 is related to other mechanisms involving PI3’K. Significant inhibitory effect of LY294002 on tissues treated with indomethacin and PGs was determined using 2-way ANOVA on repeated measures (P<0.05).
Resistance (Ω cm²)

Add PGs

Isc (µA/cm²)

Time (minutes)
Figure 11. Effect of inhibition of phosphatidylinositol-3-kinase (PI3’K) on urea-induced recovery. Treatment of ischemia-injured tissues with 200mOsm urea on the mucosal stimulated recovery of TER, as in previous experiments. However, tissues pre-treated with the PI3’K inhibitor LY294002 (10µM) failed to recover when subjected to mucosal urea, suggesting an important role for PI3’K in osmotic load-stimulated mucosal recovery.
Figure 12. Immunoblots for tight junction proteins. Tissues were subjected to a 120-minute *in vitro* recovery period, including control tissue (cont), and ischemic injured tissues treated with indomethacin (indo, 5µM), indomethacin and PGs (1µM), or indomethacin, PGs and wortmannin (wort, 10nM). Epithelium was isolated from mucosa prior to Western blotting. A: Representative Western blot for claudin-5 (a 22 KDa protein located between molecular weight markers for 31.6 and 17.8 KDa) showed evidence of reduced expression of claudin-5 in ischemia-injured epithelium treated with indomethacin compared to other treatments. B: Occludin is a protein of variable molecular weight depending upon the degree of phosphorylation, typically in the range of 65-75KDa (Wong 1997). There are no apparent differences in expression of occludin (the multiple bands located between molecular weight markers for 79 and 41 KDa) between the different treatment groups.
Figure 13. Immunofluorescence microscopic evaluation of ischemia-injured tissues for occludin. A. Normal mucosa has evidence of accumulation of occludin at the lateral membrane of cells, particularly toward the apical surface of the epithelium where inter-epithelial junctions reside (arrows). B. Ischemia-injured mucosa following a 120-minute in vitro recovery period in the presence of indomethacin (5µM). Note the disorganized appearance of occludin fluorescence, with a lack of accumulation of occludin at the region of the inter-epithelial junctions. C. Tissues treated with PGs (1µM) have a pattern of occludin fluorescence that resembles that of normal tissues (arrows). D. Tissues pre-treated with indomethacin and wortmannin (10nM) and subsequently treated with PGs have poorly organized occludin fluorescence similar to that of tissues treated with indomethacin alone. 1cm bar = 5µm.
Figure 14. Immunofluorescence microscopic evaluation of ischemia-injured tissues for ZO-1. Normal mucosa has ZO-1 exclusively localized to the region of the tight junction (A), while ischemia-injured mucosa exposed to indomethacin (5µM) for 120-minutes has evidence of diffuse ZO-1 fluorescence at the apical region of recovering epithelial cells (B). Treatment of ischemia-injured tissues with indomethacin and PGs (1µM) for 120-minutes restores localization of ZO-1 to the tight junctions (C), an effect that is inhibited by pre-treatment of tissues with wortmannin (D). 1cm bar = 5µm.
Figure 15. Proposed model of prostaglandin signaling pathways. We have previously shown that PGE₂ interacts with EP₂ and EP₃ receptors, which would be expected to activate protein kinase A (PKA) or stimulate increases in Ca²⁺ᵢ respectively (Blikslager et al. 2001). These second messengers would then phosphorylate apical Cl⁻ and Na⁺ channels, in the case of PKA, or result in opening of basolateral K⁺, in the case of increased Ca²⁺ᵢ levels. The combined effect of these second messenger-ion channel interactions is secretion of Cl⁻ and blockade of Na⁺ absorption, which we believe, results in a mucosal-to-serosal osmotic gradient (Blikslager et al. 1999). The effects of PGE₂ can be enhanced with additional application of PIG₂, which appears to activate cholinergic nerves (Blikslager et al. 1997), and genistein, which directly enhances Cl⁻ secretion via the CFTR (Blikslager et al. 2000). As shown in the present studies, addition of PGE₂ and PGI₂ to the serosal surface of ischemia-injured mucosa, or application of artificial osmotic gradients to the mucosal surface of tissues stimulates recovery of TER. These effects are prevented with application of PI3’K inhibitors, suggesting this enzyme is downstream of secretory and osmotic processes. Furthermore, we have shown that PGs restore inter-epithelial junctional localization of occludin and ZO-1, which is inhibited by the PI3’K blocker wortmannin, suggesting possible mechanisms whereby PGs may stimulate tight junction recovery following ischemic injury.
Chapter 3

The Role of Rho Kinase and Protein Kinase C in Recovery of Barrier Function in Ischemia-Injured Porcine Ileum

Dianne Little, Adam J. Moeser, Jenna G. Wooten, Anthony T. Blikslager

From the Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina.
Introduction

An intact intestinal epithelial barrier is essential for absorption of nutrients and water from the intestinal lumen, and to prevent potentially pathogenic luminal microorganisms and their products from gaining access to the systemic circulation (Podolsky, 1999). Rapid restoration of barrier function after disruption by any one of a variety of insults is critical to the innate immune response, and occurs through the coordination of villus contraction, epithelial restitution, compensatory cell proliferation, extracellular matrix and tight junction remodeling, collapse of the lateral intercellular space and closure of the tight junction (Gookin et al. 2003, Podolsky, 1999). We have previously shown that prostaglandins (PGs) are critical for recovery of intestinal barrier function after ischemic injury, not by enhancing epithelial restitution, or villus contraction, but by initiating Cl− secretion, leading to osmotically driven collapse of the lateral paracellular space and rearrangement of the tight junction proteins ZO-1 and occludin to close the tight junction (Blikslager et al. 1997, Little et al. 2003).

The Rho-family of small GTPases are critical to the organization of the actin cytoskeleton, and are responsible for retention of the sodium-hydrogen exchanger-3 (NHE3) in the apical cell membrane; inhibition of Rho results in redistribution of ezrin and actin and internalization of NHE3 away from the apical membrane (Hayashi et al. 2004). Furthermore, Rho and its downstream effector Rho kinase (ROCK) is critical for assembly and maintenance of tight junction proteins within their membrane microdomains, via interaction with the actin cytoskeleton (Nusrat et al. 2001, Walsh et al. 2001).
Of the twelve distinct isoforms of PKC, 10 are known to be expressed in intestinal epithelial cells and many isoforms have been found to be involved in regulation of the tight junction (Farhadi et al. 2006). For example, PKCα regulates tight junction permeability in a small intestinal cell line, (Marano et al. 2001) and PKCβ1 protects against oxidant-induced damage in Caco-2 cells (Banan et al. 1999). PKCδ activation is associated with disruption of tight junction integrity in intestinal epithelial cells exposed to oxidants (Banan et al. 2002). However, much of the literature has focused on the role of PKC in disruption of tight junction function, and relatively little work has focused on the role of PKC in repair of the tight junction after injury. There is recent evidence to suggest cross-talk between the Rho and Protein kinase C (PKC) pathways (Stamatovic et al. 2006). For example, PKCδ was found to be downstream of Rho/ROCK in PKD mediated protection against apoptosis following oxidant injury (Song et al. 2006).

We have previously shown that stimulation of PKC activity with the agonist phorbol myristate acetate (PMA) does not simulate PG mediated recovery of intestinal barrier function, (Blikslager et al. 2000) but in the present studies we sought to determine the role of specific PKC isoforms in PG-mediate recovery of intestinal barrier function after ischemic-injury, and to determine the role, if any of Rho/ROCK signaling in this recovery.

Our data show that both ROCK and PKCδ signaling is required for PG-mediated recovery of epithelial barrier function in ischemic-injured porcine ileal mucosa.
Materials and Methods

Experimental animal surgeries

All studies were approved by the North Carolina State University Institutional Animal Care and Use Committee. Six to eight-week-old Yorkshire crossbred pigs of either sex were housed singularly, and maintained on a commercial pelleted feed. Pigs were held off feed for 12 hours prior to experimental surgery. General anesthesia was induced with xylazine (1.5 mg/kg, IM), ketamine (11 mg/kg, IM) and thiopental (15mg/kg, IV) and was maintained with intermittent infusion of thiopental (6-8mg/kg/hr). Pigs were placed on a heating pad and ventilated with 100% O$_2$ via a tracheotomy using a time-cycled ventilator. The jugular vein was cannulated, and was used to administer Lactated Ringer’s solution intravenously at a maintenance rate of 15ml/kg/hr during anesthesia. The ileum was approached via a ventral midline incision. Ileal segments were delineated by ligating the intestinal lumen at 10-cm intervals. Loops were randomly designated as control or ischemic loops. The latter were subjected to ischemia by occluding the local mesenteric blood supply for 45-minutes.

Ussing chamber studies

Following the ischemic period, the mucosa was stripped from the seromuscular layer in oxygenated (95% O$_2$/ 5% CO$_2$) Ringer’s solution, and mounted in 3.14 cm$^2$ aperture Ussing chambers, as described in a previous study (Argenzio and Liacos, 1990). Tissues were bathed on the serosal and mucosal sides with 10ml Ringer’s solution. The serosal bathing solution contained 10mM glucose, and was osmotically balanced on the mucosal side with 10mM mannitol. Bathing solutions were oxygenated (95% O$_2$/5% CO$_2$), 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 (\( \Omega \cdot \text{cm}^2 \)) 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 \( \mu \text{A} \) for 5 seconds and the PD recorded. Short-circuit current and PD were recorded every 15-minutes for 180-minutes.

*Experimental treatments*

Tissues were bathed in Ringer's containing 5\( \mu \text{M} \) indomethacin to prevent PG production while stripping mucosa from the seromuscular tissues, and indomethacin was added to the serosal and mucosal bathing solutions in the same concentration prior to mounting tissues on Ussing chambers. Other treatments that were added to the serosal and mucosal bathing solutions prior to baseline electrical measurements were *Clostridium difficile* Toxin A (1-100ng/ml) (mucosal side only), Y27632 (1\( \mu \text{M} \)), staurosporine (100nM), tamoxifen (0.1-1\( \mu \text{M} \)), rottlerin (5-50\( \mu \text{M} \)), Gö6983 (10-100nM), Gö6976 (10-100nM) and porcine transforming growth factor-\( \beta 1 \) (0.5-50ng/ml) (serosal side only). Baseline electrical readings were taken for 30-minutes, after which 1\( \mu \text{M} \) 16,16-dimethyl-PGE\(_2\) and 1\( \mu \text{M} \) carbacyclin (the stable analog of PGI\(_2\)) were added to the serosal bathing solution. Each experimental treatment was performed on tissue from 6 pigs. All chemicals were obtained from Sigma except Gö6983 and Gö6976 which were obtained from Calbiochem.
**Gel electrophoresis and Western blotting**

Ileal mucosal scrapings from control and ischemia-injured mucosa treated with indomethacin (5μM), indomethacin (5μM) and PGs (1μM) or indomethacin (5μM), PGs (1μM) and various inhibitors applied to the Ussing chamber for 180-minutes were snap frozen and stored at -80°C prior to SDS-PAGE. One gram tissue aliquots were thawed to 4°C and added to 3 ml of chilled radioimmunoprecipitation assay buffer [0.15 M NaCl, 50 mM Tris (pH 7.2), 0.5% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 1% NP-40], including protease inhibitors (PMSF, sodium orthovanadate and aprotinin). The mixture was homogenized on ice and then centrifuged twice at 10,000 g for 10 min at 4°C, and the supernatant was saved. Protein analysis of extracted aliquots was performed using the Lowry assay to determine protein concentration of each sample. Normalized concentrations of protein extracts were mixed with SDS-PAGE sample buffer and reducing agent and boiled for 5 minutes at 100°C. Lysates were loaded on a 4 -12 % gradient pre-cast Bis-Tris polyacrylamide gels (Bio-Rad, Herclues, CA) and electrophoresis was carried out according to standard protocols. Proteins were transferred to a nitrocellulose membrane (Hybond ECL; Amersham Life Science, Birmingham, UK) by use of an electroblotting transfer apparatus according to the manufacturers’ protocol. Membranes were boiled for 5 minutes in PBS then blocked for 16 hours at 4°C in Tris-buffered 150mM NaCl solution (TBS) and 5% dry powdered milk. Membranes were washed and incubated for 2 hours in primary antibody (rabbit PKCα polyclonal; Abcam, Cambridge, MA, rabbit PKCδ polyclonal; Abcam, Cambridge, MA, rabbit PKC polyclonal; Santa Cruz Biotech, Santa Cruz, CA, rabbit Hsp70(Hsp72) polyclonal; Stressgen, Victoria, BC, Canada, rabbit ROKα/ROCK-II
polyclonal; Upstate Cell Signaling, Lake Placid, NY.) After being washed the membranes were incubated with horseradish peroxidase-conjugated secondary antibody. After additional washes, the membranes were developed for visualization of proteins by addition of enhanced chemiluminescence reagent (Amersham, Piscataway, NJ).

Data analysis
Data were reported as mean ± SE. All data were analyzed using a 2-way ANOVA or paired t-test (Sigmastat, Jandel Scientific, San Rafael, CA). A Tukey’s test was used to determine differences between treatments following ANOVA. P < 0.05 was considered significant for all analyses.

Results
Application of 1μM 16,16-dimethyl PGE2 and 1μM carbacyclin (a stable analog of PGI2) to mucosal sheets of porcine ileum injured by 45-minutes of ischemia and bathed in 5μM indomethacin resulted in recovery of TER, where as ischemia-injured tissues treated with indomethacin alone displayed minimal recovery of TER (Figure 1A). As we have shown in previous studies by Blikslager et al. (2000), this increase in TER is preceded by a sharp elevation in Isc (Figure 1B), attributable to an increase in Cl– secretion (Blikslager et al. 1999). Contrary to our expectation that treatment of the mucosal surface of ischemia-injured ileum with the Rho inhibitor Clostridium difficile Toxin A (CdTA) would inhibit PG-mediated recovery of TER, we found no effect of CdTA on PG-induced elevation in Isc (Figure 1B), and no effect of 1ng/ml or 10ng/ml CDTA on PG-mediated recovery of TER.
CdTA (100ng/ml) reduced the minimal recovery of TER in ischemia-injured tissues treated with indomethacin alone (Figure 1A) suggesting a non-specific effect on barrier function. We therefore explored the effect of inhibition of a downstream effector of Rho, the target of CdTA. Y27632, a specific inhibitor of Rho kinase significantly inhibited PG-mediated recovery of intestinal barrier function in ischemia-injured mucosa treated with indomethacin (Figure 2A), with no effect on Isc (Figure 2B).

In further studies, we attempted to elucidate the reason for the failure of CdTA to inhibit PG-mediated recovery of intestinal barrier function. Johal et al. (2004) found TGF-β1 was the predominant TGF-β isoform expressed in T84 cells treated with ≤10ng/ml CdTA, and that this TGF-β expression was protective against CdTA mediated loss of barrier function. Therefore, we applied recombinant porcine TGF-β1 (0.5-50ng/ml) to ischemia-injured porcine ileum treated with indomethacin, prior to treatment with PGs, to determine if treatment with TGF-β1 would further enhance TER. TGF-β1 treatment had no effect on TER (Figure 3A), but significantly reduced the PG-mediated elevation in Isc (Figure 3B) at the lowest dose of TGFβ1 (0.5ng/ml).

Expression of HSP72 is protective against CdTA-mediated cell damage in T84 cells (Lui et al. 2003), therefore we evaluated expression of HSP72 in control and ischemia-injured porcine ileum immediately following ischemia, and after 180 minutes in vitro recovery of ischemia-injured mucosa in Ussing chambers. There were low basal levels of HSP72 in both control and ischemia-injured ileum immediately following ischemia, but after 180 minutes of
*in vitro* incubation of tissues, ischemia-injured mucosa treated with indomethacin but not control mucosa, demonstrated upregulation of HSP72 expression (Figure 4). Treatment with PGs or CdTA did not increase expression further, but combined treatment with both PGs and CdTA resulted in further upregulation (Figure 4).

Blikslager *et al.* (2000) demonstrated that ischemia-injured porcine ileum bathed in indomethacin treated with the PKC agonist PMA had no effect on TER either in isolation or when combined with treatment with the Cl secretagogue genistein. However, the role of PKC inhibition on PG-mediated recovery of epithelial barrier function has not been explored. Treatment of ischemia-injured porcine ileum bathed in indomethacin with the non-specific PKC inhibitors staurosporine or tamoxifen had no effect on PG-mediated elevation in TER (Figure 5A) or Isc (Figure 5B). However, when rottlerin (5 x 10^{-6}M), the specific inhibitor of PKCδ was applied to similar tissue, there was significant inhibition of the PG-mediated elevation in TER (Figure 6A), but no effect on Isc (Figure 6B). The alternate inhibitor of PKC α, β, γ, δ, Gö6983 (1 x 10^{-8}M)(Figure 7), and of PKC α, β1, Gö6976 (1 x 10^{-8}M) (Figure 8) had no significant effect on PG-mediated elevation in TER, or on Isc, although there was a trend for inhibition of PG-mediated recovery of TER with Gö6983 (1 x 10^{-8}M).

When the tissue was treated with elevated doses of rottlerin (5 x 10^{-5}M), Gö6983 (1 x 10^{-7}M), Gö6976 (1 x 10^{-7}M), a reduction in resistance of indomethacin treated ischemia-injured mucosa was noted (data not shown) therefore the effects if any of these doses on PG specific pathways could not be determined, and were likely indicative of non-specific effects.
Expression of PKC was elevated in ischemia-injured indomethacin-treated mucosa, and in ileum additionally treated with PGs after 180 minutes of *in vitro* recovery, when compared to control ileum at the same time point, and when compared to control or ischemia-injured ileum at the end of the ischemic period (Figure 9A). However, elevation of PKC expression was not uniform across all isoforms. Expression of PKCα was reduced during the period of *in vitro* incubation, compared to control and ischemia-injured mucosa immediately after the end of ischemia (Figure 9B). More importantly, treatment of ischemia-injured indomethacin-treated mucosa with prostaglandins resulted in increased expression of PKCδ by 180 following ischemia (Figure 9C). Furthermore, additional treatment of PG-treated mucosa with the ROK inhibitor Y27632 reduced expression of PKCδ (Figure 9C). Treatment of PG-treated mucosa with rottlerin did not markedly alter PKCδ expression, when compared to expression of PKCδ in tissue treated with PGs alone. As expected, application of rottlerin or Y27632 to PG-treated tissue did not alter expression of PKCα (Figure 9B). In further experiments, we evaluated ROK expression in control and ischemia-injured mucosa and found it to be expressed at low levels within the mucosa, and found that expression was not changed with ischemic-injury (Figure 10A), nor was expression altered by treatment with indomethacin, PGs, or the ROK inhibitor Y27632 or rottlerin (Figure 10B).
Discussion

CdTA treatment of indomethacin-ischemia-injured tissues did not reduce the PG-mediated recovery of TER. This response was unexpected, since CdTA is known to increase tight junction permeability via disruption of F-actin and the cytoskeleton (Hecht et al. 1988). However, in the study by Hecht et al. (1988), low doses of CdTA (0.7-7ng/ml) actually increased TER, and reduction in TER was not observed until T84 monolayers were incubated with 70ng/ml CdTA. The data indicating elevation in TER at low doses of CdTA were not investigated further in the study by Hecht et al. (1988). However, the profile of cytokine expression changes with level of T84 cell exposure to CdTA (Johal et al. 2004). At doses of CdTA ≤10mg/ml, T84 cells increase expression of TGF-β1, but at doses >10ng/ml CdTA, IL-8 expression is elevated, whereas TGF-β1 expression remains at control levels (Johal et al. 2004). Furthermore, pre-treatment with recombinant TGF-β isoforms was protective against CdTA-induced disruption of epithelial barrier function. We applied recombinant porcine TGF-β1 to indomethacin-ischemia injured mucosa, prior to treatment with PGs to determine if this would result in further elevations in TER. However it did not, and furthermore, this maneuver reduced the PG-mediated elevation in Isc.

HSP72 is induced in epithelial cells in response to heat stress, oxidant injury and ischemia (Lui et al. 2003). Therefore our finding that expression of the molecular chaperone HSP72 was induced by in vitro treatment of ischemia-injured mucosa with indomethacin for 180 minutes but that 45 minutes of ischemia alone did not induce HSP72 expression were somewhat surprising, particularly given that control tissue subjected to 180 minutes of in
vitro recovery in the Ussing chamber did not demonstrate a similar response. In gastric mucosa, HSP72 expression in mucosal epithelial cells is induced by treatment with acetylsalicylic acid (Jin et al. 1999), suggesting that treatment with the non-selective cyclooxygenase inhibitor indomethacin may have induced HSP72 in ischemia-injured porcine ileum. HSP72 protects against CdTA mediated epithelial cell disruption and increased paracellular permeability, through stabilization of the actin cytoskeleton, protection against mitochondrial damage, and protection against ATP depletion (Lui et al. 2003). The further upregulation of HSP72 that occurred when indomethacin-ischemia injured tissues were treated with both PGs and CdTA suggests that this may be the mechanism of the apparent lack of effect of CdTA in the current study, even though HSP72 binds, but does not prevent RhoA glucosylation by CdTA (Lui et al. 2003).

CdTA treatment of guinea pig ileum results in Cl⁻ secretion (Moore et al. 1990), an effect not observed in this study. However the dose of CdTA used in the study by Moore et al. (1990) was 5μg/ml, 50-fold higher than the highest dose observed in the current study. Nonetheless, any agent which acts to stimulate Cl⁻ secretion may increase the osmotic load within the lumen and contribute to collapse of the lateral intercellular space and closure of the tight junction (Little et al. 2003).

Treatment of indomethacin-ischemia injured tissue with the specific ROCK inhibitor Y27632 partially inhibited PG-mediated elevation in TER, without effect on the Isc. These data are consistent with the finding of Walsh et al. (2001) that ROCK was critical for assembly of the
tight junction and recovery of barrier function in T84 monolayers in a calcium
depletion/repletion model via disruption or reassembly of the apical F-actin cytoskeleton.
The expression of ROCK is ubiquitous (Noma et al. 2006); therefore it is not surprising that
expression in this study per se did not change with ischemia, indomethacin or PG treatment,
particularly given the critical role of ROCK in transmission of ‘on/off signals’ generated by
the Rho-GTPases (Hopkins et al. 2003). The fact that ROCK inhibition did not change Isc
despite partial inhibition of PG-mediated elevations in TER suggests that ROCK is
downstream of Cl− secretion in the pathway of PG-mediated recovery of intestinal barrier
function.

In oxidant-injured injury models, increased PKCδ translocation causes increased expression
of inducible nitric oxide synthase and production of nitric oxide, which results in disruption
of the cytoskeleton and increased paracellular permeability (Banan et al. 2002, Banan et al.
2003). However, this work evaluated the role of PKCδ in disruption of the tight junction in
response to injury, and not in recovery of the tight junction after injury. In the current study,
we found that PKCδ activity is required for maximal PG-mediated elevation in TER in
porcine indomethacin-ischemia injured ileum based on results obtained with rottlerin
(5x10^{-6}M) treatment, but not for PG-mediated elevations in Isc and that PKCδ expression is
increased in indomethacin-ischemia injured tissue when additionally treated with PGs. We
also found that upregulation of PKCδ expression was reduced in PG-treated tissues
additionally treated with the ROCK inhibitor Y27632, suggesting that ROCK may play a
critical role in expression of signaling molecules required for tight junction reassembly. The
interaction of PKCδ and ROCK with both the cytoskeleton and junctional proteins critical to tight junction function, and with other signaling molecules already found to be critical to recovery of tight junction function after ischemic-injury should be further evaluated.
References


Blikslager AT, Roberts MC, Young KM, Rhoads JM, and Argenzio RA. (2000) Genistein augments prostaglandin-induced recovery of barrier function in ischemia-injured porcine


Figure 1: In initial experiments to evaluate Rho and ROCK, we used the Rho inhibitor *Clostridium difficile* Toxin A (CdTA). Treatment with CdTA (1-100ng/ml) had no effect on PG induced recovery of barrier function. Values represent mean ±SEM, n=6. A: Percent change in TER seen over 150 minutes B: Change in Isc in the 15 minute period after addition of PGs * Significant difference to ischemic-indomethacin. P≤0.05. (II=ischemic-injured porcine ileum bathed in indomethacin, PGE₂/Carbacyclin = II tissues additionally treated with prostaglandins)
Figure 2: The specific Rho Kinase inhibitor Y27632 (10^{-6} M) significantly inhibited PG induced recovery of barrier function, with no effect on Isc. Values represent mean ±SEM, n=6.

A: Percent change in TER seen over 150 minutes  
B: Change in Isc in the 15 minute period after addition of PGs. * Significant difference to ischemic-indomethacin; # significant difference to ischemic indomethacin PGs, P≤0.05
Figure 3: TGFβ1 (0.5, 5, 50ng/ml) had no significant effect on PG mediated recovery of TER but reduced the increase in Isc at 0.5ng/ml. Values represent mean ± SEM, n=6

A: Percent change in TER seen over 150 minutes after PGs were added.
B: Change in Isc in the 15 minute period after PGs were added. * Significant change to ischemic-indomethacin. # Significant change to ischemic-indomethacin and TGFβ1(0.5ng/ml) PGs. P<0.05
Figure 4: HSP72 expression in control (Cont) and ischemia-injured (Isch) tissues immediately after the end of ischemia (Time =0) and after 180 minutes of *in vitro* recovery. HSP72 expression increased in ischemia-indomethacin (Isch Indo) treated tissue, compared to control; combined treatment with PGs (PGE2/Carbacyclin) and CdTA increased this expression further.
Figure 5: Treatment with the non-specific PKC inhibitors tamoxifen (0.1-1mM) or staurosporine (100nM) does not block PG-induced recovery of TER, but treatment with 0.1mM tamoxifen does partially inhibit PG-induced elevation in Isc. Values represent mean ± SEM, n=6. A: Percent change in TER seen over 150 minutes after PGs were added. B: Change in Isc in the 15 minute period after PGs were added. * Significant difference to ischemic-indomethacin and ischemic-indomethacin PGs tamoxifen 100μM. # Significant difference to ischemic-indomethacin. P≤0.05
Figure 6: The selective PKCδ inhibitor Rottlerin (5x10^{-6}M) partially blocked recovery of barrier function stimulated by PGs (10^{-6}M), but had no effect on short-circuit current (Isc). Values represent mean ± SEM, n=6 A: Percent change in TER seen over 150 minutes after PGs were added. B: Change in Isc in the 15 minute period after PGs were added. * Significant difference to ischemic-indomethacin # Significant difference to ischemic-indomethacin PGs. P<0.05
Figure 7: There was a trend toward blockade of recovery of barrier function with the PKC \( \alpha_{\beta \gamma \delta} \) inhibitor \( \text{Gö6983} \ (1 \times 10^{-8} \text{M}) \). Values represent mean ± SEM, \( n=6 \). A: Percent change in TER seen over 150 minutes after PGs were added. B: Change in Isc in the 15 minute period after PGs were added.* Significant difference to ischemic-indomethacin. \( P \leq 0.05 \)
Figure 8: There was no significant effect of the PKCα and β1 inhibitor Gö6976 (1x10-8M) on recovery of barrier function. Values represent mean ± SEM, n=6 A: Percent change in TER seen over 150 minutes after PGs were added. B: Change in Isc in the 15 minute period after PGs were added. * Significant difference to ischemic-indomethacin. P≤0.05
Figure 9: Expression of total PKC (A) increases over a 180 minute in vitro recovery period, but expression of PKCα decreases (B). Expression of PKCδ (C) increases in PG treated ischemia-indomethacin tissue, after 180 minutes of in vitro recovery. Treatment with the ROK inhibitor Y27632 reduces this upregulation. (C=control tissue)
Figure 10: Expression of ROKα/ROCK-II does not change with ischemia in the three representative pigs evaluated (A) or with in vitro recovery and treatment with ROK or PKCδ inhibitors (B).
Chapter 4

Cyclooxygenase and its inhibitors – an evolving field.

Dianne Little
**History of cyclooxygenase discovery**

“*An aspirin a day doubles the chances of a long life*” (Vane 2003).

For over 3500 years salicin, the active principle of the common white willow (*Salix abla*) has been used to treat pain, rheumatism and fever (Vane 2000). When salicylic acid was first synthesized in Germany in 1874 (Vane and Botting 2003), the race for development of similar drugs began. By 1899, the pharmaceutical manufacturing house of Frederick Bayer in Germany had named and released the more palatable acetylsalicylic acid (aspirin) onto the market, with claims of its antipyretic, analgesic and anti-inflammatory properties (Vane 2000). The average person today still consumes around 80 aspirin tablets per year (Vane 2000). Despite active research efforts and the release of similar compounds onto the market, the mechanism of action of aspirin-like drugs remained elusive for many years. A major break-through occurred in the 1960s with the discovery and characterization of a group of lipid-derived chemical mediators: the prostaglandins (PGs) (Vane 2000). At around the same time, ‘rabbit aorta contracting substance’ (RCS) was discovered, (subsequently identified as thromboxane A<sub>2</sub> (TXA<sub>2</sub>)) and the ability of acetylsalicylic acid to block RCS release from guinea pig lungs during anaphylaxis was identified. The ability of acetylsalicylic acid and indomethacin, but not morphine, hydrocortisone or mepyramine (a histamine H<sub>1</sub> reverse agonist) to block PG production was discovered shortly thereafter (Vane 1971). Cyclooxygenase, the enzyme responsible for production of prostaglandin H<sub>2</sub> (PGH<sub>2</sub>) from arachidonic acid (Figure 1) and the target of the new class of non-steroidal anti-inflammatory drugs (NSAIDs) was discovered in 1976 (Hemler *et al.* 1976). A second cyclooxygenase isoform was
discovered in 1991, and was designated COX-2, the original isoform being COX-1 (Xie et al. 1991). A third isoform, designated COX-3 was identified in 2002, and is a splice variant of COX-1 that retains intron 1 (Chandrasekharan et al. 2002). The same group identified a second splice variant of COX-1, designated Partial COX-1a or PCox-1a. This variant not only retains intron 1, but also contains a deletion of exons 5-8. Since then, other intron 1 retaining splice variants of COX-1 have been identified and shown to have biological function (Qin et al. 2005). The biological function of these splice variants is under debate, but there is evidence to suggest that transcription of these splice variants occurs in response to osmotic stress in intestinal epithelial cells, and that the RNA transcripts of these variants regulate COX-1 and COX-2 mRNA (Nurmi et al. 2005). To further complicate matters, several splice variants of COX-2 have also been discovered (Davies et al. 2004). However, the number of known splice variants of COX-1 is greater than for COX-2. Some of these COX-1 splice variants are projected to code for heme-binding proteins, but the definitive role of COX splice variants in health or disease is not yet known (Roos and Simmons 2005).

**Patterns of cyclooxygenase expression.**

COX-1 is expressed constitutively in most tissues and elaborates prostanoids involved in normal physiological processes. In the gastrointestinal tract, PGE$_2$ and prostacyclin (PGI$_2$) reduce gastric acid secretion, increase the production of protective mucus, vasodilate mucosal blood vessels, and increase duodenal bicarbonate secretion. While these effects are largely beneficial, there is evidence to suggest that active COX-1 may be
detrimental in certain circumstances. For example vasodilation of gastric blood vessels increases reperfusion injury after ischemia (Hiratsuka et al. 2005). Although COX-1 is not typically upregulated in inflammatory conditions, there is evidence to suggest that expression of COX-1 is increased in the lamina propria mononuclear cells of gastric mucosa with increasing severity of mucosal ulceration (Bhandari et al. 2005). In the kidney, during acute allograft rejection after transplantation, COX-1 is upregulated in blood vessels and infiltrating interstitial cells (Hoffman et al. 2006). The mechanisms of COX-1 upregulation have been substantially less well studied than the mechanisms of COX-2 upregulation, despite the importance of COX-1 inhibition as a target of cardiovascular therapy. However, Tomlinson et al. (2004) found that expression of both COX-1 and COX-2 isoforms are increased in equine jejunal mucosa, immediately after the end of 2 hours of ischemia. Furthermore, COX-1 expression is upregulated in ovarian cancer, and several cell lines increase expression of COX-1 when stimulated with phorbol-esters; several transcription factor binding sites have been identified as part of these studies (DeLong and Smith 2005). Further work is required to elucidate the pathways by which COX-1 expression is regulated in non-malignant states, and is likely to be forthcoming given the increasing number of conditions in which upregulation of expression of COX-1 appears to occur.

Local in vivo COX-2 expression is upregulated in inflammation, ischemia and in other inflammatory conditions, is stimulated by pro-inflammatory cytokines and mitogens, and is pivotal in the pathogenesis of cancer. For example, PGE₂ elaborated by COX-2
increases tumor cell proliferation and inhibits apoptosis (Kashfi and Rigas, 2005). Early work evaluating the function and distribution of COX-2 suggested that there was no constitutive expression, but this has subsequently been found to be an oversimplification. For example in the gastrointestinal tract, constitutive expression of COX-2 has been identified in the mouse proximal colon (Porcher et al. 2004). In the rat, the highest level of COX-2 expression in the intestine is observed at the ileocecal junction, in the lamina propria of the apical villus rather than the epithelial cells themselves (Haworth et al. 2005). Inhibition of COX-2 delays healing of gastric ulcers and may exacerbate colitis (Reuter et al. 1996, Schmassmann et al. 1998).

The population of prostaglandins produced by cyclooxygenase depends not only on expression or upregulation of the specific COX-1 or COX-2 isoform, but also on the expression and localization of specific prostaglandin synthases. For example an inducible membrane-associated form of PGE$_2$ synthase is coupled to COX-2, but the constitutively expressed cytosolic PGE synthase is coupled to COX-1 (Murakami et al. 1999). The activity of specific prostaglandin synthases determines in large part whether increased expression of COX-2 is beneficial or detrimental. For example, in a model of cardiac inflammation, induced by endotoxemia, PGE$_2$ synthase and COX-2 expression both peak by 4 hours after endotoxin administration, but PGD$_2$ synthase expression does not peak until 48 hours after endotoxin administration (Schuligoi et al. 2005). PGE$_2$ contributes to inflammation and hyperalgesia, therefore inhibition of PGE$_2$ production would seem to be beneficial, however, the PGD$_2$ metabolite, 15-deoxy-delta$^{12,14}$ PGJ$_2$ is
not only a potent agonist of peroxisome proliferator-activated receptor gamma (PPARγ) but can act independently of PPARγ to inhibit PGE₂ synthase directly in rat chondrocytes, thus blocking production of pro-inflammatory prostaglandins, and can also inhibit activation and nuclear translocation of NFκB in the chondrocyte (Bianchi et al. 2005, Straus and Glass, 2001). These studies suggest that COX-2 inhibition during the resolution phase of inflammation may be detrimental, because synthesis of valuable anti-inflammatory prostaglandins is inhibited.

**Non-steroidal anti-inflammatory drugs: cyclooxygenase inhibition.**

The NSAIDs inhibit the active site of cyclooxygenase through hydrogen bonding to arginine at amino acid 120. At positions 434 and 523, critical for the difference in specificity of COX-1 and COX-2 inhibitors, COX-1 has isoleucine residues and COX-2 smaller valine residues. The smaller size of the valine residues in COX-2 permits access to a side-pocket, which is the site of binding of COX-2 selective drugs. Furthermore, at amino acid 513, COX-2 has a charged arginine residue and COX-1 an aromatic histidine residue. The larger isoleucine residues of COX-1 at these sites blocks entrance to the side pocket (Bertolini et al. 2001). Binding of COX inhibitors to the active site of the membrane bound cyclooxygenase isoenzymes prevents cycling of the membrane derived arachidonic acid through the ‘U’ shaped active site of the cyclooxygenase enzyme, and prevents formation of the aromatic ring that characterizes the prostanoids (Bertolini et al. 2001).
Non-steroidal anti-inflammatory drugs: cyclooxygenase independent effects.

Just as the cyclooxygenase gene and its expression are far more complex than originally discovered and a constantly evolving field of investigation, the mechanisms of action of cyclooxygenase inhibitors are proving to be equally complex. Much of this work was initiated when several epidemiological and clinical studies demonstrated the role of cyclooxygenase inhibitors in the prevention of cancer, and found that COX-2 is upregulated in many types of neoplasia (Kulp et al. 2004). For example, celecoxib, a selective COX-2 inhibitor was recently approved by the United States Federal Drug Administration for its demonstrated efficacy as an adjuvant treatment of familial adenomatous polyposis, a condition in which up-regulation of COX-2 is well recognized (Steinbach et al. 2000). Active research in the field of chemo-prevention is targeting discovery of alternative drugs to NSAIDs to provide the benefits of these drugs on prevention of cancer, without the deleterious side-effects of long-term administration of NSAIDs on the gastrointestinal tract or on the cardiovascular system. Therefore there is much active research to determine the non-cyclooxygenase mechanisms of action of cyclooxygenase inhibitors.

In cell lines with no cyclooxygenase expression, both the non-selective NSAIDs indomethacin, suldinac, piroxicam and ibuprofen, and the selective COX-2 inhibitor, NS-398 had anti-neoplastic and anti-proliferative effects (Zhang et al. 1999). Additionally there is evidence from other areas of investigation to suggest that non-cyclooxygenase targets may play an important role in the mechanism of action of the NSAIDs. Salicylic
acid has anti-inflammatory properties, even though it does not possess the acetyl group of acetylsalicylic acid, and therefore does not inhibit cyclooxygenase by acetylation (Tegeder et al. 2001). At therapeutic concentrations, S-flurbiprofen inhibits cyclooxygenase, but R-flurbiprofen, the other enantiomer of flurbiprofen lacks cyclooxygenase activity and therefore is unable to block PGE$_2$ release in a rat model of paw inflammation. Nonetheless, R-flurbiprofen is almost as effective as dexamethasone at reducing inflammation (Geisslinger et al. 1994). As a result of this and other work, multiple non-cyclooxygenase targets of cyclooxygenase inhibitors have been identified.

The major non-cyclooxygenase targets of the cyclooxygenase inhibitors of particular relevance to inflammation, and therefore of potential relevance to recovery of intestinal barrier function after injury are phosphatidylinositol 3’-kinase (PI3K)/Akt signaling, uncoupling of oxidative phosphorylation, PPAR$\gamma$, nuclear factor $\kappa$B (NF$\kappa$B), the mitogen activated protein kinases (MAPKs), and the heat shock protein response.

**Phosphatidylinositol 3’-kinase (PI3’K)/Akt signaling**

PI3’K/Akt signaling is the final common pathway of a number of cytokine and tyrosine kinase pathways and is key in the regulation of cell proliferation, transcription, translation, growth and apoptosis (Osaki et al. 2004, Zhu et al. 2004). Dysregulated upregulation of Akt is key for three critical characteristics of neoplasia: angiogenesis, tumor invasiveness, and proliferation (Di Cristofano and Pandolfi, 2000). Furthermore, dysregulation of this pathway has also been implicated in autoimmune diseases and in
diabetes mellitus (Osaki et al. 2004). Celecoxib is a direct inhibitor of 3-phosphoinositide-dependent kinase-1 (PDK-1), an upstream regulator of Akt signaling and therefore this pathway is a major non-cyclooxygenase target of celecoxib that explains the antiproliferative effects of this drug on cancer cells (Kulp et al. 2004, Zhu et al. 2004). IκB kinase is a downstream effector of Akt (Figure 2). Phosphorylation of IκB kinase phosphorylates IκB, which leads to nuclear translocation of NFκB and transcription of NFκB genes, including cox-2. Therefore inhibition of PDK-1 by celecoxib results ultimately in down-regulation of COX-2 expression. Rofecoxib, another COX-2 selective coxib drug has no effect on PDK-1 activity (Patel et al. 2005), suggesting that each NSAID should be evaluated carefully in the circumstances in which it will be used for its cyclooxygenase independent effects.

In a mesangial cell line however, celecoxib increased activation of the PI-3’K pathway and phosphorylation of Akt leading to phosphorylation of JNK, a mitogen-activated protein kinase (MAPK) (Hou et al. 2005). Not surprisingly, downstream effectors of JNK, c-Jun and ATF-2 also demonstrated increased phosphorylation after treatment of mesangial cells with celecoxib, effects that could be blocked with specific inhibition of JNK (Hou et al. 2005). These effects on the Akt/PI3’K pathway were indirect however, since celecoxib treatment enhanced oxygen free radical generation, which in turn leads to increased phosphorylation of Akt and activation of the PI3’K pathway. Specific inhibition of PI3’K leads to a reduction in JNK phosphorylation and reduced expression
of hemoxygenase-1 (HO-1), reversing the effects of celecoxib treatment on HO-1 expression. Elevations of HO-1 expression caused by celecoxib treatment would be expected to increase heme degradation, and increase the production of reaction products including carbon monoxide, biliverdin and iron. Carbon monoxide and the other products of heme degradation have important antioxidant, anti-inflammatory and cytoprotective functions and are protective in ischemia/reperfusion injury and inflammation in the intestine (Attuwaybi et al. 2004, Deshane et al. 2005). Therefore from these studies, celecoxib may be expected to be protective against ischemia/reperfusion injury.

The effect of NSAIDs on Akt activity in different cell lines is particularly interesting in relation to the effect of NSAIDs on intestinal repair and differentiation. Akt expression is elevated in cells that are fully differentiated, compared to cells in a less differentiated state (Li X et al. 2004). In intestinal epithelial cells, both PI3’K and Akt are associated with lipid rafts, microdomains of the membrane that are enriched in glycosphingolipids and cholesterol. Lipid rafts cluster cell surface receptors, membrane proteins, and signaling molecules within a small defined area (Simons and Toomre, 2000). PI3’K and Akt are associated with the sodium hydrogen exchanger NHE3 within the lipid rafts, and act to increase brush border sodium absorption in the absence of PTEN (Li X et al. 2004). PI3’K is required for prostaglandin-mediated recovery of barrier function in ischemia-injured porcine ileum, and inhibition retards normal formation of the tight junction after ischemic-injury (Blikslager et al. 1999, Little et al. 2003).
The role of specific NSAIDs on this pathway in relation to recovery of intestinal barrier function after ischemic injury may be worthy of further investigation, particularly given the very specific effects of a given NSAID on different cell lines.

**Uncoupling of oxidative phosphorylation.**

Substantial work has focused on the role of cyclooxygenase inhibition on accumulation of arachidonic acid accumulation and on uncoupling of mitochondrial oxidative phosphorylation, particularly in the post-ischemic heart. In cardiac tissue, arachidonic acid accumulates during reperfusion injury (van der Vusse et al. 1997). In addition, COX inhibition blocks eicosanoid production and leads to further elevations in levels of arachidonic acid. In the presence of COX inhibition, arachidonic acid metabolism is redirected to the lipoxygenase and cytochrome P450 (epoxygenase) pathways (Figure 1) to produce alternate arachidonic acid eicosanoids (Heindl and Becker 2000, Yu et al. 2006). Elevated arachidonic acid levels also inhibit mitochondrial oxidative phosphorylation and increase generation of reactive oxygen species (Fosslien 2005). Furthermore, several NSAIDs, including indomethacin, aspirin, diclofenac, the coxibs, meloxicam and the selective COX-2 inhibitor SC-236 directly uncouple or inhibit mitochondrial oxidative phosphorylation (Fosslien 2005, Krause et al. 2003, Petrescu and Tarba 1997, Tibble et al. 2000). The effects of cyclooxygenase inhibitors on coupling of oxidative phosphorylation are tissue and dose dependent. For example, celecoxib does not uncouple oxidative phosphorylation in the rat small intestine in contrast to
indomethacin (Tibble et al. 2000). At clinically relevant concentrations meloxicam and piroxicam have no effect on oxidative phosphorylation, and do not uncouple oxidative phosphorylation until concentrations are approximately three-times the therapeutic anti-inflammatory serum concentrations (Moreno-Sanchez et al. 1999). The effect of cyclooxygenase inhibitors such as acetylsalicylic acid on oxidative phosphorylation is related to their structure rather than their effects on cyclooxygenase, and requires a carboxylic acid group. In contrast, neither piroxicam nor meloxicam have a carboxylic acid group (Heindl and Becker, 2001). Furthermore, as a result of uncoupling of oxidative phosphorylation, ATP production from mitochondria is reduced and intestinal permeability increases (Tibble et al. 2000).

Further work focusing on the effects of structure and physico-chemical properties of the cyclooxygenase inhibitors on their non-cyclooxygenase effects and pro- or anti-oxidant capacity was initiated when the COX-2 selective coxib drug, rofecoxib but not celecoxib was found to increase the risk of atherothrombotic cardiovascular events (Walter et al. 2004). The COX-2 selective coxib drugs are divided into two classes, the sulfones (rofecoxib, etoricoxib) and the sulfonamides (celecoxib, valdecoxib). The sulfone coxibs reduce the low-density lipoprotein (LDL) antioxidant capacity of human plasma, by acting as pro-oxidants whereas the sulfonamide coxibs, and meloxicam, diclofenac, naproxen and ibuprofen do not. The pro-oxidant activity of the sulfone coxibs occurs through increased rate of free-radical production via a non-COX dependent mechanism.
Furthermore, the sulfone coxibs increase levels of isoprostanes (free-radical prostaglandin isomers generated non-enzymatically from arachidonic acid), and change the electron density patterns within the phospholipid bilayers whereas the sulfonamide coxibs do not (Walter et al. 2004).

**Peroxisome proliferator-activated receptor activity**

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors of the nuclear receptor family (Issemann and Green, 1990). They bind to sequence specific DNA response elements as a heterodimer with the retinoic acid receptor and thereby exert their effect on target gene transcription (Figure 3) (Kliewer et al. 1994). PPARγ can also inhibit gene transcription through the NFκB, STAT and AP-1 signaling pathways (Chinetti et al. 2000). There have been three isoforms of PPAR isolated: PPARα, PPARβ and PPARγ (Delerive et al. 2001). PPARα is expressed in hepatocytes, the proximal tubule cells of the kidney, and in cardiomyocytes. PPARγ is expressed in adipocytes and enterocytes, and PPARβ is expressed almost ubiquitously (Kersten et al. 2000). The roles of PPARα, PPARβ in inflammation have been less well studied than the role of PPARγ. PPARγ expression is down-regulated by lipopolysaccharide, TNFα, IL-1 and IL-6. In contrast IL-4 induces PPARγ expression (Chinetti et al. 2000). PPARγ inhibits the expression of inducible nitric oxide synthase, and blocks the actions of several pro-inflammatory cytokines, such as TNFα, IFNγ,
TGFβ, IL-1 and IL-6 (Jaradat et al. 2001). In smooth muscle cells, PPARγ inhibits COX-2 expression, but in mammary epithelial cells PPARγ increases COX-2 transcription (Chinetti et al. 2000). These paradoxical results could be explained by differences in kinetics between the NFκB pathway and the classic PPAR response element (PPRE) (Chinetti et al. 2000). Recent work has demonstrated the importance of PPARγ in inflammation and in intestinal ischemia/reperfusion injury. PPARγ knock-out mice demonstrate exacerbated gastric ischemia/reperfusion injury compared to wild type mice. Additionally, PPARγ activation reduced the severity of gastric ischemia/reperfusion injury, and blocked the upregulation of intercellular adhesion molecule-1 (ICAM-1), and tumor necrosis factor (TNFα) via inhibition of IκB degradation and inhibition of NFκB activation (Boyault et al. 2004, Nakajima et al. 2001). PPARγ directly inhibits NFκB through binding of the p65 subunit of NFκB (Figure 3) (Chen F et al 2003). PPARγ contains a consensus site for the MAPKs which when phosphorylated results in inhibition of PPARγ activity, thus providing an important means of regulating PPARγ during the pro-inflammatory state (Figure 3) (Adams et al. 1997).

PPARγ agonists are divided into two classes, the thiazolidinediones (TZDs) and the non-TZDs (Feinstein et al. 2005). The non-TZDs include several long chain fatty acids, 15-deoxyΔ^{12,14}PGJ₂, and certain NSAIDs. 15-deoxyΔ^{12,14}PGJ₂ attenuates the severity of gastric ischemia-reperfusion injury in rats via a PPARγ-dependent mechanism (Takagi et
Regardless of the potentially detrimental effects of COX-2 inhibition in the later stages of inflammation on 15-deoxy Δ^{12,14} PGJ₂ induced inhibition of NFκB and PGE₂ synthase activity discussed earlier, certain cyclooxygenase inhibitors are able to directly activate PPARγ. For example, the COX-2 selective inhibitor celecoxib directly activates PPARγ in renal mesangial cells, an effect that is blocked by albumin. Furthermore, in this study, albumin blocked the effects of the natural ligand 15-deoxy Δ^{12,14} PGJ₂ on PPARγ activity (López-Parra et al. 2005). However, in tissues surrounded by interstitial fluid, celecoxib is likely to activate PPARγ without the inhibitory effects of albumin. In order of potency, naproxen, indomethacin and ibuprofen were all found to activate PPARγ at doses relevant to COX-1 and COX-2 inhibition (Jaradat et al. 2001). Fenoprofen and flufenamic acid also bind and activate PPARγ, but with less potency than indomethacin (Lehmann et al. 1997). In contrast, acetylsalicylic acid and sodium salicylate have no effect on PPARγ activity, and sulindac decreases PPARγ activity (Tegeder et al. 2001).

In Caco-2 intestinal epithelial cells, the NSAIDs mefenamic acid, meclofenamate, NS-398 (a specific COX-2 inhibitor), sulindac, flurbiprofen and ibuprofen treatment all resulted in upregulation of COX-2 protein and mRNA expression, but had no effect on COX-1 expression suggesting a transcriptional rather than a translational effect. Treatment with indomethacin, piroxicam, naproxen, aspirin or ketorolac did not change COX-1 or COX-2 expression. COX-2 transcription was dependent on the presence of the
PPRE, lending support to the hypothesis that certain NSAIDs modulate COX-2 transcription via a PPARγ dependent mechanism (Meade et al. 1999). In a rodent model of intestinal ischemia/reperfusion, NS-398 not only reversed the deleterious effects of COX-2 activity on inflammation, injury and impaired transit, but induced expression and nuclear translocation of PPARγ, suggesting that administration of COX-2 specific NSAIDs with PPARγ ligand activity may be protective in intestinal ischemia/reperfusion (Sato et al. 2005). Interestingly, intraluminal glutamine increased PPARγ activity, and was protective in intestinal ischemia/reperfusion injury suggesting an alternate mechanism of restoring intestinal function after ischemia/reperfusion (Sato et al. 2005 B).

Given that 15-deoxy Δ^{12,14} PGJ_2 is a more potent PPARγ agonist than the NSAIDs investigated so far, particularly at doses sufficient for cyclooxygenase inhibition it is possible that the agonist effect of NSAIDs on PPARγ activity is insufficient to offset the reduction in PPARγ activity caused by inhibition of 15-deoxy Δ^{12,14} PGJ_2 synthesis by the NSAIDs (Tegeder et al. 2001). Further evaluation of the interaction between 15-deoxy Δ^{12,14} PGJ_2, the NSAIDs, and PPARγ is required.
**NFκB activity**

NFκB is rapidly activated in response to a variety of pro-inflammatory stimuli, and is the driving force for the induction of a wide variety of genes involved in the inflammatory cascade through binding of NFκB subunits to DNA. Deletion of IKK prevents the systemic inflammatory response that typically results in multiple organ dysfunction syndrome after intestinal ischemia/reperfusion injury, but deletion of IKK from enterocytes results in dramatic increase in rate of apoptosis of enterocytes from reperfused intestinal mucosa, hence demonstrating a critical role for NFκB in causing multiple organ dysfunction syndrome following ischemia/reperfusion injury, and also a critical role in protecting enterocytes from apoptosis in response to environmental stressors (Chen LW et al. 2003).

Many NSAIDs can influence the NFκB pathway via their interactions with Akt/PI3’K, but sodium salicylate and aspirin are the best studied of the NSAIDs in relation to their direct effect on NFκB activity. Both aspirin and salicylate inhibit lipopolysaccharide or cytokine induced activation of NFκB by preventing IκB phosphorylation and degradation, though these effects were only seen at the upper limits of anti-inflammatory and therapeutic doses (Tegeder et al. 2001). Ibuprofen, sulindac, flunixin meglumine and flurbiprofen all also inhibit NFκB activation, but indomethacin, ketoprofen and ketorolac do not (Ahn and Aggarwal, 2005; Tegeder et al. 2001). Ibuprofen and sulindac act via a
similar mechanism as salicylate to inhibit IκB, but flurbiprofen does not. Mechanism of action of flurbiprofen appears to be via direct interaction with NFκB or by interaction with nuclear chaperones such as HSP 70, which facilitate nuclear translocation of NFκB (Tegeder et al. 2001). Meloxicam and indomethacin both block lipopolysaccharide-induced increases in NFκB activation in murine peritoneal macrophages (Hu et al. 2001). Meloxicam was also found to inhibit adhesion of polymorphonuclear leukocytes to human synovial cells and ICAM-1 expression via inhibition of NFκB activity (Li LC et al. 2002). Rofecoxib inhibited NFκB activation in lipopolysaccharide treated murine macrophages and reduced inducible nitric oxide synthase and COX-2 expression (Callejas et al. 2003). In PMA-stimulated human monocytes, however rofecoxib did not inhibit TNFα release from activated human monocytes, even though NFκB activation was also inhibited (Lavagno et al. 2004). In TNF-α stimulated tumor cells, aspirin< ibuprofen< sulindac< phenylbutazone< naproxen< indomethacin< diclofenac< celecoxib in increasing order of potency inhibited IKK, and NFκB regulated COX-2 expression (Takada et al. 2004).

In contrast, in TNF-α stimulated H-29 cells, increased levels of NFκB translocation were identified after simultaneous treatment with diclofenac, compared to TNF-α stimulation alone, but diclofenac by itself had no effect on NFκB translocation (Poligone and Baldwin 2001). In addition, high doses of celecoxib cause activation of NFκB
translocation rather than inhibition (Niederberger et al. 2001). Incubation of hepatocytes with salicylate, aspirin, indomethacin, ibuprofen or a rofecoxib derivative did not inhibit NFκB translocation or expression of NFκB dependent genes, such as inducible nitric oxide synthase (Callejas et al. 2002). These data suggest that NSAIDs may have differential effects on NFκB depending on the degree of cell stimulation or differentiation, and on the cell line evaluated.

Cyclopentenone prostaglandins such as 15-deoxy Δ^{12,14} PGJ₂ and PGA₁ and PGA₂ elaborated during arachidonic acid metabolism also inhibit NFκB via PPARγ-independent mechanisms involving direct inhibition of IKK activation (Figure 3), or direct binding to target DNA sequences of NFκB, thereby inhibiting NFκB binding (Straus et al. 2001). In contrast, PGE₂ enhances NFκB activity (Poligone and Baldwin, 2001). These findings collaborate well with the role of COX-2 in inflammation, an early pro-inflammatory activity and a later anti-inflammatory response discussed earlier.

**The mitogen activated protein kinases (MAPK)**

There are three major MAPK cascades that lead to altered gene expression: ERK 1/2, JNK and p38 MAPK. They are activated by a variety of cytokines, and pathogenic stimuli, such as lipopolysaccharide. The p38 MAPK pathway is critical for stabilization
of many pro-inflammatory mRNA transcripts such as COX-2 mRNA. Furthermore, MAPK frequently phosphorylate and activate cytoplasmic transcription factors, leading to increases in gene transcription. However, activation of transcription factors such as NFκB is not required for p38 MAPK induced upregulation of COX-2 expression in response to lipopolysaccharide in intestinal epithelial cells, and p38 MAPK can activate the Cox-2 gene promoter directly (Grishin et al. 2006).

In some cases, salicylate and aspirin can block activation of ERK-1 and ERK-2, and some COX-2 selective drugs can also inhibit ERK-2. Salicylate may also activate both p38 and JNK in certain circumstances (Tegeder et al. 2001). Indomethacin treatment results in prolonged activation of the MAPK in colon cancer cells (Kim et al. 2002). Other than those mentioned there appear to be relatively few studies that evaluate specifically the effects of the NSAIDs on either MAPK expression or activity. The cyclopentenone prostaglandin 15-deoxy Δ12,14 PGJ2 can activate JNK, which leads to activation of transcription factors such as c-Jun leading to activation of AP-1 mediated transcription (Straus and Glass 2001). The MAPK are however intimately involved in recovery of epithelial barrier function after ischemic injury. Shifflett et al. (2004) found that inhibition of p38 MAPK or ERK 1/2 both inhibited recovery of ischemia-injured porcine ileum, but that only inhibition of p38 MAPK blocked upregulation of COX-2 that occurred in ischemic injury. Administration of exogenous PGs ameliorated the response of the injured tissue to p38 or ERK 1/2 inhibition and permitted recovery of intestinal
barrier function. This work also found that JNK negatively regulated COX-2 expression in ischemia-injured intestine. When similar tissue was treated with the non-selective cyclooxygenase inhibitor indomethacin prior to treatment with the specific ERK 1/2 inhibitor PD-98059, recovery of barrier function as assessed by transepithelial electrical resistance (TER) (Figure 4A) was further enhanced by the addition of exogenous PGs, caused by further stimulation of short circuit current (Isc) than that caused by addition of PGs alone (Figure 4B), suggesting that p38 MAPK has an additional role in recovery of intestinal barrier function as a downstream effector of prostaglandins. Addition of the specific p38 MAPK inhibitor to control tissue treated with indomethacin had no effect on intestinal barrier function or on short circuit current (Figure 5 A&B).

**The heat shock protein response**

Heat shock proteins are a well-conserved group of transcriptional activators that are essential for cell survival in response to a variety of external stressors. Many of the heat shock proteins also function as chaperones for the folding, sorting and assembly of proteins. The heat shock response is particularly important for example in cellular response to ischemia/reperfusion, and in the rat gastric mucosa, Heat shock protein (HSP) 70, 27, 86, 90, 60 and 8 gene expression are all upregulated after ischemia and reperfusion (Naito *et al.* 2005). Pre-treatment of HeLa cells with indomethacin reduces the time and temperature required for induction of the heat shock response through reducing the threshold of heat shock factor-1 (HSF-1) activation and binding to DNA;
HSF-1 is a major transcription factor for the heat shock proteins, and acts as an inducer of heat shock genes and inhibitor of cytokine gene expression (Locke et al. 2002). Both indomethacin and ibuprofen cause HSP 70 to localize to the nucleus (Lagunas et al. 2004). Sodium salicylate activates HSF1, and aspirin, ibuprofen and piroxicam can induce HSP 70 mRNA expression (Housby et al. 1999, Tegeder et al. 2001). The role of the heat shock proteins in recovery of mucosal barrier function after ischemic injury needs further evaluation.

To summarize, the concept that the NSAIDs are pure cyclooxygenase inhibitors is overly simplistic, and consideration should be given to other actions that they may have in any given experimental model or signaling pathway. Further research is required to elucidate the non-cyclooxygenase effects of cyclooxygenase inhibitors in veterinary medicine and consideration should be given to these factors when considering the therapeutic and adverse effects of the NSAIDs.
References:


*Biochem Biophys Res Commun.* **338:**53-61.

*Acta Biochim Pol.* **52:**273-84.

*Cell.* **100:**387-90.


Figure 1: Pathways of arachidonic acid metabolism. PG ‘X’₂ synthase, ‘X’ represents D,E,F,I or TXA₂.
Figure 2: Interaction of the phosphatidylinositol-3 kinase (PI3K) pathway with the NFκB pathway via Akt, and the interaction of selected COX-2 inhibitors with this pathway.
Figure 3: PPARγ activation blocks NFκB nuclear translocation, but active PPARγ binds retinoid X receptor (RXR) and other transcriptional co-activators and binds to the specific PPAR response element (PPRE) in the promoter of the target gene.
Figure 4: Recovery of indomethacin treated ischemia-injured porcine ileum and response to prostaglandins in the presence or absence of the specific ERK 1/2 inhibitor PD-98059 as assessed by recovery of transepithelial electrical resistance (A) or response of short circuit current (Isc) (B) to prostaglandin administration over time in the Ussing chamber.
Figure 5: Response of control tissue treated with indomethacin to specific inhibition of ERK 1/2 as assessed by transepithelial electrical resistance (A) and short circuit current (Isc) (B) over time after mounting in the Ussing Chamber.
Chapter 5

The Effects of the Cyclooxygenase Inhibitors Meloxicam and Flunixin Meglumine on Recovery of Ischemic-Injured Equine Jejunum.

Dianne Little, S. Aubrey Brown, Nigel B. Campbell, Adam J. Moeser, Jennifer L. Davis, Anthony T. Blikslager.
**Introduction**

Colic is the leading cause of death in horses after old age (Traub-Dargatz *et al.* 2001, Wineland 1998). Many of these deaths are attributable to the results of increased intestinal absorption of endotoxin through a compromised intestinal epithelial barrier (Moore *et al.* 1981). Many of the systemic effects and clinical signs of endotoxemia are mediated by prostaglandins (PGs), which are elaborated from arachidonic acid by the constitutively expressed cyclooxygenase-1 (COX-1) and the inducible cyclooxygenase-2 (COX-2) enzymes. Therefore non-steroidal anti-inflammatory drugs (NSAIDs) such as flunixin meglumine are frequently administered to horses with colic to inhibit COX and ameliorate the detrimental effects of prostaglandins. However, prostaglandins are critical for recovery of barrier function in ischemia-injured intestine (Blikslager *et al.* 1997, Campbell and Blikslager 2000). Treatment of ischemia-injured equine jejunum with the non-selective COX inhibitor flunixin meglumine retards recovery of intestinal barrier function (Tomlinson *et al.* 2004, Tomlinson and Blikslager 2004, Tomlinson and Blikslager 2005). Previous evaluation of the COX-2 selective inhibitors deracoxib and etodolac in the horse (Tomlinson *et al.* 2004, Tomlinson and Blikslager 2005) have not resulted in identification of an alternative NSAID to flunixin meglumine that is practical to use in the clinical case of colic in the horse.

Meloxicam is a preferential COX-2 inhibitor in the enolic acid class of NSAIDs with a favorable gastrointestinal side-effect profile in humans compared to non-selective cyclooxygenase inhibitors (Gates *et al.* 2005). The European Agency for the Evaluation of
Medicinal Products (2006) recently approved meloxicam for oral and intravenous use in the horse, at a dose of 0.6mg/kg bwt q24 hours. Our hypothesis was that administration of the preferential COX-2 inhibitor meloxicam to horses subjected to jejunal ischemia would permit sufficient local PG production to permit recovery of intestinal barrier function, while ameliorating clinical signs of pain and endotoxemia attributable to the detrimental systemic effects of PGs.

Furthermore, we speculated that the NSAIDs flunixin meglumine and meloxicam would affect expression or activity of other genes associated with the inflammatory response to ischemia-reperfusion, given the recent interest in cyclooxygenase-independent actions of cyclooxygenase inhibitors on the mitogen activated protein kinases (MAPKs) and peroxisome proliferator-activated receptor gamma (PPARγ) (Tegeder et al. 2001).

Materials and Methods

All procedures were approved by the North Carolina State University Animal Care and Use Committee. Eighteen horses with no previous history of systemic disease, colic or abdominal surgery aged 3-20 years and weighing 350-700kg, were included in the study. The animals had previously undergone a 4-week quarantine period including vaccination, anthelmintic therapy and observation. An i.v. catheter was placed in the left jugular vein and each horse was assigned to one of three treatment groups (groups 1,2 and 3). Each horse was
administered ceftiofur sodium (2.2mg/kg bwt i.v.) once prophylactically. Five minutes prior
to pre-medication, horses in group 1 received 12ml 0.9% NaCl i.v. then every 12 hours for
the duration of the study, horses in group 2 received flunixin meglumine (1.1mg/kg bwt i.v.)
then every 12 hours, and horses in group 3 received meloxicam (0.6mg/kg bwt i.v.) and
immediately prior to euthanasia, approximately 21 hours later. The dose of meloxicam was
determined on the basis of previously published pharmacokinetic studies in the horse
(Toutain and Cester, 2004) and on the dose licensed by the European Agency for the
Evaluation of Medicinal Products. Venous blood was sampled for pharmacokinetic analysis
5 minutes, 30 minutes, and 3, 9 and 19 hours after meloxicam was administered. Each horse
was pre-medicated with xylazine (1.1mg/kg bwt i.v.), and induction of general anesthesia
was achieved with diazepam (0.1mg/kg bwt i.v.) and ketamine (2.2mg/kg bwt i.v.). After
orotracheal intubation horses were maintained at a surgical plane of anesthesia with
isoflurane vaporized in 100% O₂. All horses received butorphanol tartrate (0.05mg/kg bwt
i.v.) immediately after positioning in dorsal recumbency. A midline celiotomy was
performed using aseptic technique and the distal jejunum located. The contents of the distal
jejunum and ileum were milked manually into the cecum, after which two 30cm loops of
jejunum were isolated by use of Doyen intestinal forceps. The loops were approximately 1m
apart, and the first was 60cm orad to the anti-mesenteric band of the ileum. One loop in each
horse served as control tissue and one was subjected to 2h ischemia, created by clamping
local mesenteric arteries and veins, using Penrose drains to avoid excessive trauma to the
vessels. At a site 1m proximal to the most proximal isolated loop, a 30cm section of jejunum
was manipulated by milking the intestine fifty times with dry surgical gauze. The site of
manipulation was marked with cruciate sutures placed at the anti-mesenteric border. At the end of ischemia, the loops were reperfused, and a full thickness wedge biopsy was obtained from control and ischemic loops. The biopsy sites and celiotomy incision were closed routinely and horses were allowed to recover from general anesthesia. Horses were monitored according to standard hospital protocol post operatively, and all horses received butorphanol tartrate (0.05mg/kg bwt i.m.) every 4 hours. In addition, horses were monitored 2, 8 and 16 hours after the end of ischemia for pain using a modification of a previously established behavioral pain scoring system (Table 1) (Pritchett et al. 2003). Horses had access to water ad libitum immediately after surgery and were offered small amounts of timothy hay post-operatively. Horses were euthanatized with sodium pentobarbital (100mg/kg bwt i.v.) 18 hours after the end of ischemia. After euthanasia, ischemia-injured, control and manipulated jejunal tissues were harvested for in vitro experiments and immediately placed in oxygenated (95% O2 and 5% CO2) equine Ringer’s solution (NaCl 114mM, KCl 5mM, CaCl$_2$ 1.25mM, MgCl$_2$ 1.10mM, NaHCO$_3$ 25mM, NaH$_2$PO$_4$ 0.3mM, Na$_2$HPO$_4$ 1.65mM).

**Ussing Chamber Studies**

Harvested jejunum was incised along the anti-mesenteric surface and mucosa was stripped from the seromuscular layer in oxygenated equine Ringer’s solution and mounted in 3.14cm$^2$ aperture Ussing chambers as described previously. Mucosa was bathed in 10ml oxygenated equine Ringer’s solution on both mucosal and serosal sides maintained at 37°C by the use of circulating water-jacketed reservoirs. The serosal bathing solution additionally contained
10mmol/l glucose and was osmotically balanced on the mucosal side with 10mmol/l mannitol. The spontaneous potential difference (PD) was measured by use of Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through silver-silver chloride electrodes with a voltage clamp that corrected for fluid resistance. Resistance ($\Omega \cdot \text{cm}^2$) was calculated from the spontaneous PD and short-circuit current ($I_{\text{SC}}$). If the spontaneous PD was between -1mV and 1mV, tissues were current clamped at $\pm 100 \, \mu\text{A}$ for 5 seconds and PD recorded. $I_{\text{SC}}$ and PD were recorded every 15 minutes for 2 hours. Data were entered into spreadsheets that calculated transepithelial electrical resistance (TER) from $I_{\text{SC}}$ and PD by use of Ohm’s law. Results were expressed as the mean TER ± SEM over a 1-hour period used for inulin and LPS-FITC fluxes after a 30 minute equilibration period.

**Inulin Flux**

$^{14}$C-methylated inulin (10$\mu$Ci/ml) in 5% inulin was placed on the mucosal side of control and ischemia-injured tissues mounted in Ussing chambers following an initial 30-minute equilibration period. One 60-min flux was subsequently conducted from 30 to 90 minutes of the experimental period using samples obtained from the serosal side of the bathing solution and counted for $\beta$ emission (counts/min) in a scintillation counter. Mucosal-to-serosal flux ($J_{\text{ms}}$) of inulin was calculated by using standard equations.
**FITC-LPS Flux**

Lipopolysaccharide (LPS) from Escherichia coli serotype O111:B4 labeled with 3μg fluorescein isothiocyanate/mg LPS was placed on the mucosal side of control and ischemia-injured tissues mounted in Ussing chambers (2.5×10⁻⁸g FITC-LPS/ml Ringer’s solution) following an initial 30-minute equilibration period. One 60-min flux was subsequently conducted from 30 to 90 minutes of the experimental period using 200 μl samples obtained in triplicate from the serosal side of the bathing solution. Fluorescence of samples and a standard curve created for each experiment were measured in a fluorescent 96-well plate reader at an excitation wavelength of 485nm, and emission of 538nm. Mucosal-to-serosal flux (Jₘs) of LPS was calculated by using standard equations.

**Histological examination**

Mucosa from control and ischemia-injured jejunum was obtained by wedge biopsy at the end of ischemia and at the time of euthanasia, 18 hours after the end of ischemia. Three micrometer thick sections were cut at 300μm intervals from each mucosal sample and processed for routine hematoxylin and eosin light microscopy. Sections were independently examined by 2 different observers (SAB & DL). Three well-oriented villi were identified in each section. The height of the villus and the width at the midpoint of the villus were obtained by 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, an 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 by using a modified formula for the surface area of a cylinder. Villus surface area = \((2\pi.1/2 [(4/\pi)d]h)\), where \(\pi = 3.14\), \(d\) = villus diameter at midpoint, and \(h\) = villus height. 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 percentage of denuded villous surface area was used as an index of epithelial restitution. Number of epithelial neutrophils were counted in a 10\(\mu\)m\(^2\) grid reticle on 5 different villus tips, and expressed as mean count per mm\(^2\).

**Meloxicam sample analysis**

Plasma samples were obtained immediately prior to drug administration, 5 and 30 minutes and 3, 9, and 19 hours after meloxicam administration for estimation of meloxicam concentrations in plasma and were analyzed by high pressure liquid chromatography (HPLC) using a method developed in our laboratory. The HPLC system was an Agilent 1100 series with a variable wavelength ultraviolet detector set at 365 nm. Sample separation was achieved using a Zorbax RX-C18 4.6mm x 150mm reverse phase column. The mobile phase was 60% 0.05M sodium acetate buffer and 40% HPLC grade acetonitrile. Glacial acetic acid was added to decrease the pH of the mobile phase to 3.7. Meloxicam sodium standard (93.8% pure) was dissolved in deionized water to a total concentration of 1 mg/mL meloxicam base. Further dilutions were made in deionized water to create spiking solutions used to make standard curves in pooled blank equine plasma prior to each run. All samples were subjected to solid phase extraction using Oasis HLB 1 mL cartridges in a vacuum manifold. Cartridges were initially conditioned with 1 mL HPLC grade methanol and 1 mL
deionized water. Plasma (1 mL) was extracted through the cartridge that was then washed with a 95:5 (v/v) mixture of deionized water and methanol. The sample was then eluted into clean borosilicate glass tubes using 1 mL of methanol and evaporated under compressed room air at 40°C for 25 minutes. The sample was reconstituted with 200 µL of mobile phase for injection onto the HPLC system. The injection volume was 25 µL and the retention time was approximately 4.5 minutes.

Pharmacokinetic analysis

The data were analyzed using a computer program. Noncompartmental analyses were performed to determine pharmacokinetic parameters including the plasma concentration at time zero (Cp₀), elimination half-life (T½), clearance, apparent volume of distribution and area under the curve extrapolated to infinity (AUC₀-∞).

Gel electrophoresis and Western blotting.

Mucosal scrapings from control and ischemia-injured mucosa of three representative horses from each drug treatment group obtained 18-hours after the end of ischemia at the time of euthanasia were snap frozen and stored at -80°C. One gram tissue aliquots were thawed to 4°C and added to 3 ml of chilled radioimmunoprecipitation assay buffer [0.15 M NaCl, 50 mM Tris (pH 7.2), 0.5% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 1% NP-40], including protease inhibitors (PMSF, sodium orthovanadate and aprotinin). The mixture was homogenized on ice and then centrifuged twice at 10,000 g for 10 min at 4°C, and the supernatant was saved. Protein analysis of extracted aliquots was performed using the Lowry
assay to determine protein concentration of each sample. Normalized concentrations of protein extracts were mixed with SDS-PAGE sample buffer and reducing agent and boiled for 5 minutes at 100°C. Lysates were loaded on a 4-12% gradient pre-cast Bis-Tris polyacrylamide gels and electrophoresis was carried out according to standard protocols. Proteins were transferred to a nitrocellulose membrane by use of an electroblotting transfer apparatus according to the manufacturers’ protocol. Membranes were boiled for 5 minutes in PBS then blocked for 16 hours at 4°C in Tris-buffered 150mM NaCl solution (TBS) and 5% dry powdered milk. Membranes were washed and incubated for 2 hours in primary antibody (1:500 dilution of anti-human COX-1, 1:500 dilution of anti-human COX-2, 1:5,000 dilution of anti-rabbit β-actin, 1:2,000 anti-rabbit PPARγ, or 1:500 anti-rabbit total p38, or overnight at 4°C for phospho-P38 (1:1000 dilution)). After being washed the membranes were incubated with horseradish peroxidase-conjugated secondary antibody at a dilution of 1:15000 (COX-1, COX-2) 1:2500 (β-actin), 1:1000 (PPARγ, phospho-P38), or 1:5000 (total p38). After additional washes, the membranes were developed for visualization of proteins by addition of enhanced chemiluminescence reagent. A membrane that was initially probed for phospho-p38 was stripped using standard procedures and reprobed for total p38 so that the ratio of phospho-p38: total p38 could be calculated and compared by densitometry using the same membrane. The same membrane was subsequently stripped a second time and reprobed for β-actin to confirm equal protein loading of lanes. Densitometry was performed by using appropriate software. Data were expressed as mean (±SEM) densitometry units for control or ischemia-injured tissue for each of the three drug treatment groups (saline, flunixin, meloxicam). In addition, densitometry of ischemia-injured tissue
was compared to control tissue from the same horse, and expressed as the mean (±SEM) percent change in ischemia-injured tissue for each drug treatment group. Phospho-p38:Total-p38 were calculated for each tissue from each horse, the ratio for control tissue from saline treated horses was corrected to a value of 1, and the ratios for the other tissue groups corrected to this value. The change in ratio for ischemia-injured tissue compared to control tissue for the same horse was then calculated and expressed as a mean change in ratio (±SEM).

Statistical Analysis
Pre-operative and post-operative pain scores, heart and respiratory rates and prostanoid levels were analysed by 2-way analysis of variance (ANOVA) for the effects of drug treatment and time. Mucosal neutrophil counts, villus height measurements, and percent of villus denuded of epithelial cells were evaluated by 2-way ANOVA for the effects of drug treatment and loop (control vs ischemia-injured). Transepithelial electrical resistance, inulin and LPS-FITC flux and densitometry data were analysed by 1-way ANOVA. Post hoc analyses were performed with Tukey’s tests for parametric data. For all tests, statistical significance was set at p<0.05.
Results

Clinical Parameters and Pain Scores (Fig 1 & 2)

No horse was deemed by the investigators to require additional analgesia, thus data from all 18 horses were available for analysis. All horses ate hay when offered postoperatively; subjectively horses treated with meloxicam had greater appetite than either saline or flunixin treated horses and horses treated with flunixin had greater appetite than horses treated with saline.

There was no significant difference in total pre-operative pain scores for the three groups of horses. Horses treated with saline alone had significantly higher total pain scores at all post-operative time points compared to pre-operative pain score and compared to horses treated with flunixin or meloxicam at all post-operative time points (2, 8 and 16 hours after the end of ischemia. There was no significant difference in total pain scores between horses administered flunixin and those administered meloxicam (Figure 1).

There was no significant difference between treatment groups for pre-operative heart or respiratory rates. Both heart and respiratory rates were significantly elevated at all post-operative time points in saline treated horses compared to pre-operative values, except for respiratory rate at 8 hours postoperatively (Figure 2). Post-operative heart rate in horses treated with flunixin was significantly increased compared to pre-operative values at 8 hours post-operatively, but was not significantly elevated in horses treated with meloxicam at any post-operative time point. Post-operative heart rates were lower in flunixin treated horses
compared to saline treated horses 16 hours postoperatively, and in meloxicam treated horses compared to saline treated horses at 8 and 16 hours post-operatively (Figure 2A). Postoperative respiratory rate was significantly lower in horses treated with meloxicam compared to those treated with saline at all time-points and at 16 hours postoperatively respiratory rate was lower for horses treated with flunixin compared to saline. Postoperative respiratory rate at 16 hours post-operatively was significantly lower in horses treated with meloxicam compared to those treated with flunixin (Figure 2B).

Transepithelial electrical resistance (TER) (Fig 3)

TER of ischemia-injured jejunum in horses treated with saline was significantly elevated compared to control uninjured tissue. In horses treated with flunixin this recovery in TER of ischemia-injured jejunum was blocked and was not significantly different from control tissue. Meloxicam treatment permitted recovery of TER in ischemia-injured tissue to a similar extent as ischemia-injured tissue from horses treated with saline. Exposure of control or ischemia-injured jejunum to FITC-LPS for measurement of mucosal-serosal flux did not change TER compared to jejunum not treated with LPS (data not shown). Furthermore manipulation alone did not change TER, when compared to control loops of jejunum for any of the drug treatment groups.
Inulin and FITC-LPS Flux (Fig 4)

Inulin demonstrated a trend (P=0.07) for increased mucosal-serosal flux across ischemia-injured tissue of flunixin treated horses compared to saline or meloxicam treated horses (Figure 4A). FITC-LPS flux was significantly greater in ischemia-injured jejunum from horses treated with flunixin compared to control loops in all three treatment groups and when compared to ischemia-injured jejunum from horses treated with meloxicam. There was a trend (P=0.375) for increased flux of FITC-LPS across ischemia-injured jejunum from horses treated with flunixin compared to similar jejunum from horses treated with saline (Figure 4B).

Histological studies (Fig 5-7)

Numbers of neutrophils were increased in ischemia-injured mucosa compared to control mucosa at the end of 2 hours of ischemia (Figure 5A). There was no difference between drug treatment groups. Eighteen hours after the end of ischemia, numbers of mucosal neutrophils were still elevated in all ischemia-injured tissue compared to control tissue, but there was an additional increase in numbers of neutrophils in ischemia-injured tissue of horses treated with flunixin or meloxicam (Figure 5B). At the end of two hours of ischemia there was significant epithelial denudation (Figure 6), and significant villus contraction (Figure 7). There was no effect of drug treatment on degree of epithelial denudation or of villus contraction at the end of ischemia. Eighteen hours after the end of ischemia, ischemia-injured mucosa continued to demonstrate significant villus contraction, and percent of each villus denuded was still elevated compared to control jejunum, but there was evidence of
epithelial restitution in ischemia-injured jejunum because a lower percent of each villus was denuded 18 hours after injury compared to immediately after the end of ischemia. Epithelial restitution was particularly advanced in ischemic tissue from meloxicam treated horses, where percent villus denudation was not different to control tissue at 18 hours after the end of ischemia (Figure 6).

*Meloxicam pharmacokinetic parameters. (Table 2, Figure 8)*

Intra-assay accuracy and precision were calculated at 10, 1 and 0.1 µg/mL. Five samples of each concentration were analyzed. Overall accuracy of the HPLC assay was within 4.04 ± 2.66% of the true value, and precision was within 4.04 ± 0.23% of the mean. Average recovery for drug in plasma at these concentrations was 81 ± 6.6%. The limit of detection was 0.019 µg/mL and the limit of quantification was 0.025 µg/mL. Calibration curves were linear between the concentrations of 10 µg/mL and 0.025 µg/mL, with a coefficient of determination ($r^2$) of > 0.99 and calibration samples within +/- 15% of the true concentration.

Pharmacokinetic parameters obtained for meloxicam (Table 2) were compared to other reported values (Lees *et al.* 1991, Toutain and Cester, 2004, Toutain *et al.* 2004). Drug clearance was much lower in this study compared to values previously reported. Plasma meloxicam concentrations obtained were typical of intravenous administration (Figure 8).
**Western Blots (Figure 9,10)**

There was no difference in β-actin expression across all lanes (including stripped blots, data not shown), confirming equal protein loading. For saline treated horses, COX-1, PPARγ, and Total P38 MAPK expression were significantly elevated in ischemia-injured mucosa, compared to control mucosa, 18 hours after the end of ischemia. There was a trend for increased expression of COX-2, and an increased ratio of phospho-p38:total p38 in ischemia-injured mucosa compared to control.

For horses treated with flunixin, COX-1 was significantly elevated in ischemia-injured mucosa, but there was a trend for the percent increase in ischemic compared to control to be less than for horses treated with saline. There was a trend for COX-2 upregulation in ischemia-injured mucosa compared to control and a trend for the increase in ischemia-injured expression compared to control to be less in flunixin treated horses than for horses treated with saline. PPARγ expression in ischemia-injured mucosa from horses treated with flunixin was significantly elevated compared to all other groups, but the percent increase in ischemia-injured mucosa compared to control was similar to saline treated horses. Total p38 expression was increased in ischemia-injured compared to control mucosa of flunixin treated horses. The increase in total p38 in ischemia-injured mucosa compared to control was less than for saline treated horses, and there was a smaller increase in the phospho-p38:total p38 ratio in ischemia-injured compared to control mucosa.
In meloxicam treated horses, COX-1 was upregulated in ischemia-injured mucosa relative to control mucosa, but there was a trend for the degree of this increase to be less than either the saline or flunixin treated horses. The same trend was evident for COX-2. PPARγ expression in both control and ischemia-injured mucosa from meloxicam treated horses was increased relative to control mucosa from saline treated horses. There was significantly less increase in PPARγ expression in ischemia-injured mucosa compared to control in meloxicam treated horses than for either saline for flunixin treated horses. Total p38 expression was increased in ischemia-injured mucosa from meloxicam treated horses, but this increase compared to control was significantly less than for horses treated with saline or flunixin. Furthermore, the ratio of phospho-p38:total p38 tended to increase in ischemia-injured mucosa, compared to control, but this increase was less than in saline treated horses, and was similar to flunixin treated horses.

**Discussion**

The effect of treatment with meloxicam on postoperative pain scores, heart rates and respiratory rates was comparable or better than the effect of treatment with flunixin meglumine, when compared to horses treated with saline, suggesting that meloxicam deserves further evaluation as an analgesic for pain caused by colic and endotoxemia in the horse.

The pre-emptive administration of NSAIDs (flunixin meglumine or meloxicam) in this study, immediately prior to induction of general anesthesia and administration of butorphanol
(0.05mg/kg bwt i.v.) immediately after induction of anesthesia allows comparison to the pain scoring data of Tomlinson *et al.* (2004) to assess the effect of pre-emptive analgesia in this model. In the study by Tomlinson *et al.* (2004) a similar dose of flunixin meglumine was administered to horses in a similar experimental protocol to the current study, and the same pain scoring system was used by similar investigators, but the first dose of flunixin was not administered until the end of 2-hours of ischemia, after approximately 150 minutes of general anesthesia, and a similar dose of butorphanol to that used in the current study was not used intramuscularly until 2-hours after recovery from anesthesia in the study by Tomlinson *et al.* (2004).

In the study by Tomlinson *et al.* (2004) at 2 hours after the end of anesthesia and 18 hours after the end of ischemia, median pain score was 17.5 (range 16-20) and 12 (range 11-16) respectively for horses treated with saline, and 12 (range 11-14) and 9 (range 9-11) respectively for horses treated with flunixin. In the current study at approximately the same time points, pain scores were considerably less; at 2 and 16 hours after the end of ischemia, median (range) pain score was 8 (6-16) and 7 (4-10) respectively for horses treated with saline, and 3 (0-8) and 1 (0-4) respectively for horses treated with flunixin. There are limited studies that evaluate and compare different perioperative analgesia techniques in the horse, particularly in relation to timing of administration of analgesics (Johnson *et al.* 1993, Raekallio *et al.* 1997). Although statistical analysis cannot be performed on these data the authors believe that these studies demonstrate the beneficial effect of pre-emptive analgesia on pain in the immediate post-operative period in the horse undergoing abdominal surgery.
Pre-emptive administration of NSAIDs in this study compared to the timing of administration of NSAIDs in previous studies did not appear to affect the results of the current mucosal studies, because there was no difference in effect of flunixin meglumine on mucosal damage, or recovery from ischemic-injury on the mucosal parameters evaluated when compared to the study by Tomlinson et al. (2004). Furthermore, it is common in the clinical setting for horses presenting with naturally occurring strangulating obstruction of the small intestine to be treated with flunixin meglumine prior to general anesthesia, correction of the strangulating lesion and subsequent reperfusion.

Flunixin administration retards recovery of TER in ischemia-injured jejunal mucosa compared to horses treated with saline, 18 hours after the end of ischemia, indicating impaired mucosal barrier function. Meloxicam administration does not impede recovery of ischemia-injured jejunum, and permits recovery of TER in ischemia-injured mucosa to a similar extent as horses treated with saline, indicating that meloxicam and saline treated horses had enhanced intestinal barrier function of ischemia-injured loops compared to flunixin treated horses. We have previously shown that administration of the non-selective cyclooxygenase inhibitor flunixin retards recovery of ischemia-injured equine jejunum, and that prostaglandins are required for recovery of barrier function, measured by TER in ischemia-injured porcine ileum (Blikslager et al. 1997; Tomlinson et al. 2004). Furthermore, treatment of ischemia-injured equine jejunum in vitro with the prostaglandin E₁ analogue misoprostol reversed the detrimental effects of flunixin on TER in vitro immediately after the end of ischemia (Tomlinson and Blikslager, 2005). Data from this
study suggests that the COX-2 preferential inhibitor meloxicam permits sufficient COX-1 activity for prostaglandin-mediated recovery of intestinal barrier function, while inhibiting the detrimental effects of COX-2 elaborated prostaglandins on clinical signs of endotoxemia and pain.

Ischemia-injured equine jejunal mucosa does not always demonstrate TER greater than that of control uninjured jejunum in the post-operative period. Immediately after the end of jejunal ischemia in the horse, TER of ischemia-injured mucosa is less than that of control jejunum (Tomlinson and Blikslager, 2004). Within 2 hours of reperfusion in vitro TER of ischemia-injured mucosa recovers significantly, but flunixin-treated ischemia-injured jejunum does not demonstrate this recovery response (Tomlinson and Blikslager, 2005). Exposure of ischemia-injured jejunum treated with flunixin to LPS in vitro immediately after the end of ischemia results in further detrimental effects on TER, whereas LPS exposure had no effect on ischemia-injured jejunum not treated with flunixin (Tomlinson and Blikslager, 2004).

In the current study, eighteen hours after the end of ischemia, TER of ischemia-injured mucosa from saline or meloxicam treated horses was greater than that of control jejunum, but the TER of flunixin treated ischemia-injured jejunum was similar to that of control jejunum. These data suggest that prostaglandins are critical for this ‘elevated’ recovery response in ischemia-injured jejunum, and that this ‘elevated’ response of TER is critical for
recovery of barrier function since LPS flux was elevated in ischemia-injured jejunum from horses treated with flunixin, but not in ischemia-injured jejunum from other groups of horses. Furthermore, 18-hours after the end of ischemia, we found that the TER of ischemia-injured jejunum from horses treated with flunixin did not change with exposure to LPS, in contrast to the situation immediately after the end of ischemia. The time of onset and the duration of this ‘elevated’ recovery of TER remain unknown. There was no difference between the degree of villus denudation or restitution in flunixin treated horses compared to saline treated horses and this is in agreement to other studies, where treatment of ischemia-injured porcine ileum with the non-selective cyclooxygenase inhibitor indomethacin does not retard epithelial restitution, compared to untreated ischemia-injured ileum (Little et al. 2003).

There are several possible explanations for the ‘elevated’ recovery of TER in ischemia-injured jejunal mucosa. Firstly, in the normal mucosa, paracellular permeability is not uniform through along the whole villus-crypt axis. Paracellular permeability is lower on the villus compared to the crypt which is relatively ‘leaky’ (Madara and Marcial, 1984). Prostaglandins mediate recovery of barrier function in ischemic-injury via collapse of the lateral intercellular space (Little et al. 2003). The presence of elevated concentrations of prostaglandins in ischemia-injured equine jejunum may contribute to collapse of the lateral intercellular space throughout the whole of the villus-crypt axis, contributing to elevations in TER above control tissue because of increased collapse of the lateral intercellular space and enhanced barrier function within the crypts. Secondly, the ‘elevated’ recovery of TER in ischemia-injured jejunum is not associated with reductions in inulin or LPS flux below that
of control mucosa, even though LPS and inulin flux increases when the ‘elevated’ recovery of intestinal barrier function is impaired, as occurs with flunixin treatment. The inter-epithelial tight junction is composed of a variety of proteins such as occludin and the claudins all of which have extracellular domains. Interactions of these proteins allow the formation of a variety of pores within the junction, which are size and charge restrictive. Thus mediators of inflammation can differentially regulate paracellular permeability and paracellular flux of molecules of a variety of sizes and charge (Watson et al. 2005), leading to the possibility that prostaglandins selectively reduce passage of small electrolytes that contribute to TER in ischemia-injured tissue, and reduce the movement of large molecules such as inulin or LPS used in flux studies through larger pores. Inhibition of prostaglandin synthesis may therefore abolish the selectivity of these pores, leading to an increased movement of electrolytes and therefore a reduction in TER, and also to increased movement of larger molecules that are typically used for flux studies. However, the exact mechanism of ‘elevated’ TER in ischemia-injured equine jejunum 18 hours after the end of ischemia awaits further mechanistic study.

The elevation of LPS flux that occurs in ischemia-injured jejunum from horses treated with flunixin is of particular concern. Attenuation of the passage of LPS across the intestinal mucosa is critical to reduce the pro-inflammatory action of LPS on the systemic inflammatory response and the deleterious effects of LPS on enterocyte activation and on epithelial restitution (Cetin et al. 2004). Intestinal epithelial cells have impaired ability to migrate in the presence of endotoxin, caused by a RhoA/phosphatidylinositol 3-kinase
dependent increase in focal adhesion formation to the underlying matrix and increased delivery of β1-integrins to the enterocyte cell membrane at the leading edge of cell migration, resulting in an increase in cell-matrix adhesion (Cetin et al. 2004, Qureshi et al. 2005). Furthermore, LPS can itself induce increases in permeability and failure of epithelial barrier function via myosin light chain kinase activation and contraction of the perijunctional actinomyosin ring resulting in opening of the tight junction and an increase in paracellular permeability (Moriez et al. 2005). It is likely that in horses with intestinal ischemia treated with flunixin, increased LPS flux across the intestinal barrier in itself contributes to sustained epithelial cell dysfunction, and further exacerbates initial inhibition of recovery of barrier function caused by inhibition of cyclooxygenase and endogenous prostaglandin production.

Several drugs, including 5-amino-salicyclic acid, an aspirin derivative, have been shown to enhance epithelial restitution and proliferation after mucosal wounding (Baumgart et al. 2005). However, NSAIDs are typically found to be detrimental to epithelial restitution, even though the predominant action of prostaglandins on recovery of mucosal barrier function is on closure of the paracellular space, and not on restitution (Gookin et al. 2003, Szabo et al. 2002). In this study, meloxicam was not protective against the degree of villus denudation at the end of ischemia, but after 18 hours of recovery, percent villus denudation in ischemia-injured mucosa from horses treated with meloxicam was not different to control mucosa, and was significantly less than ischemia-injured mucosa from horses treated with flunixin or saline, indicating that meloxicam had a positive effect on epithelial restitution. There was no
effect of meloxicam treatment on villus height, either immediately after the end of ischemia, or 18 hours later when compared to other treatment groups indicating that meloxicam did not change the degree of villus contraction that occurs in jejunal mucosa in response to injury. Reduced flux of LPS across ischemia-injured mucosa in horses treated with meloxicam compared to horses treated with flunixin may be partly responsible for the enhanced epithelial restitution, but it is possible that a non-cyclooxygenase activity of meloxicam may be involved in this enhanced restitution, given that LPS flux was not different between meloxicam and saline treated horses.

It is interesting that treatment with either meloxicam or flunixin results in a further increase in accumulation of mucosal neutrophils in ischemia-injured mucosa, compared to saline treatment, even though numbers of neutrophils in control mucosa of all three treatment groups was not different. In another study, (Tomlinson et al. 2004) there was no difference in numbers of mucosal neutrophils in ischemia-injured tissues from horses treated with saline, flunixin or etodolac, 18 hours after the end of ischemia. The difference between the two studies is likely related to the earlier administration, prior to induction of general anesthesia of flunixin or meloxicam treatment in the current study. Oral administration of meloxicam has been associated with elevated xanthine oxidase activity, and both meloxicam and the non-selective cyclooxygenase inhibitor indomethacin reduce superoxide dismutase activity (Villegas et al. 2000), suggesting that some NSAIDs may increase free radical formation during injury. Furthermore, doses of meloxicam that block PGE2 production are insufficient to block LTB4 production, a potent neutrophil chemoattractant (Engelhardt et al.)
1996). Some NSAIDs are able to increase the generation of arachidonic acid (Heindl and Becker 2000) and elevated arachidonic acid levels inhibit mitochondrial oxidative phosphorylation and increase generation of reactive oxygen species (Fosslien 2005). Given all of these factors it is conceivable that administration of NSAIDs prior to ischemic injury may exacerbate free-radical production and LTB4 concentrations during reperfusion, resulting in increased numbers of infiltrating neutrophils. This is unlikely to be important in the clinical situation, since NSAIDs are rarely administered to horses before strangulating obstruction occurs. Furthermore, epithelial barrier function in meloxicam treated horses appears to recover in a similar manner to saline treated horses, and the detrimental effects of flunixin treatment are similar that that reported previously even in the face of this increase in neutrophil influx.

COX-1 and thromboxane synthase are co-localized in the platelet, therefore activation leads to production of thromboxane A2, which is rapidly catalyzed to its more stable metabolite thromboxane B2. The IC\textsubscript{50} (drug concentration at which 50% activity is inhibited) for COX-1 is therefore determined when 50% inhibition of thromboxane B2 concentration is achieved by a given drug concentration. Determination of PGE\textsubscript{2} concentration is similarly used as a marker of COX-2 activity, since in the early phases of inflammation, prostaglandin E synthase is colocalised with COX-2, and therefore produced in larger quantities than other prostaglandins (Brideau \textit{et al.} 2001). Therefore the IC\textsubscript{50} for COX-2 is determined by 50% inhibition of PGE\textsubscript{2} concentration.
In whole blood assays on horse blood, the ratio of the IC₅₀ for COX-1 to the IC₅₀ for COX-2 for carprofen, phenylbutazone and flunixin meglumine is 1.6, 1.6, and 0.3 respectively, indicating that carprofen and phenylbutazone are essentially non-selective, and that flunixin meglumine is COX-1 selective (Brideau et al. 2001). Beretta et al. (2005) inhibited COX-1 activity with aspirin prior to evaluation of PGE2 concentrations for estimation of COX-2 activity in another equine whole blood study evaluating the cyclooxygenase selectivity of phenylbutazone, flunixin, carprofen and meloxicam and found that flunixin and phenylbutazone were COX-1 selective, while meloxicam and carprofen were weakly COX-2 selective. For meloxicam, at 50% inhibition, the IC₅₀ COX-1: IC₅₀ COX-2 was 3.8, but at 80% inhibition, the IC₈₀ COX-1:IC₈₀ COX-2 reduced to 2.2, indicating not surprisingly that meloxicam demonstrated less COX-2 selectivity at higher doses. It appears therefore that meloxicam selectivity in the horse may be less than for other species, for example in the dog, meloxicam is 10 times more selective for COX-2 than COX-1 (Brideau et al. 2001). However, whole blood assays may be of limited relevance to the COX-1 or COX-2 selectivity demonstrated at tissue level, because NSAIDs are highly bound to albumin (<95%), and in the presence of low protein concentrations, such as would occur in extracellular conditions, the potency of many drugs (diclofenac, naproxen, rofecoxib and salicylate) against COX-1 increases. However this appears to be related to chemical structure of the drug, since the potency of aspirin, celecoxib, indomethacin, lumiracoxib, meloxicam against COX-1 does not increase in the presence of low protein concentrations. Potency of drugs against COX-2 is largely unaffected by protein concentrations. (Warner et al. 2006) It is therefore likely that meloxicam retains COX-2 selectivity in mucosa, where
protein concentrations are low, but the COX selectivity of flunixin at the tissue level, particularly in circumstances where capillary permeability is increased such as endotoxemia, is unknown.

It has been suggested that the IC$_{80}$ rather than the IC$_{50}$ for COX-1 and COX-2 inhibition be used to calculate selectivity, on the basis that a valid anti-inflammatory effect is obtained at 80% inhibition of COX-2 activity (Beretta et al., 2005). The EC$_{50}$ (plasma concentration to achieve 50% of maximum stimulation) of meloxicam in a carpitis model in the horse is 0.2μg/mL (Toutain and Cester, 2004). Meloxicam has a molecular weight of 373.38, therefore the molarity of the EC$_{50}$ concentration of meloxicam is 5.36 x 10$^{-7}$M, a concentration which approximates to the IC$_{50}$ for COX-2 inhibition, and to 40% inhibition of COX-1 in the study by Beretta et al. (2005). However, in the current study, following a single intravenous dose, plasma concentrations of meloxicam remained in excess of 0.5μg/mL for approximately 13 hours after administration, a molarity of 1.34 x 10$^{-6}$ M, corresponding to approximately 65% COX-2 inhibition and the IC$_{50}$ COX-1 inhibition according to the work published by Beretta et al. (2005); mean plasma concentration did not fall to 0.2μg/mL for the duration of our pharmacokinetic study (19 hours). These data suggest that the selectivity of meloxicam in this study was somewhere between 3.806 for the IC$_{50}$ COX-1:IC$_{50}$ COX-2 and 2.239 for the IC$_{80}$COX-1:IC$_{80}$ COX-2 previously published (Beretta et al. 2005), and that some of the findings of this study may not be related to the COX-2 selectivity of meloxicam in the horse, given that at best, meloxicam is only around 3.8 times more selective for COX-2 than COX-1 in the horse.
In the current study, clearance was much slower than the values previously reported (Table 2). Therefore the dose and frequency may need to be re-evaluated in the clinical setting where factors such as dehydration, fluid therapy, concurrent drug administration, anesthesia endotoxemia and the systemic inflammatory response play an important role on glomerular filtration rate and therefore drug clearance. Only after these studies have been completed can the use of meloxicam in the treatment of colic be recommended. The low volume of distribution in this study (Table 2) is comparable to other studies, and is likely due to extensive plasma protein binding of meloxicam (Toutain et al. 2004). The terminal half life reported here was considerably shorter than that reported previously by Toutain et al. (2004), but was comparable to that reported concurrently by Toutain and Cester (2004) and by Lees et al. (1991). However, evaluation of clinical parameters of pain scores, heart rate and respiratory rate in this study suggested that once daily treatment was sufficient, and plasma concentrations remained above 0.2μg/mL for the 19 hours of the pharmacokinetic component of this study.

Tomlinson et al. (2004) found no significant elevation in COX-1 or COX-2 expression in ischemia-injured jejunum 18 hours after the end of ischemia, except in horses treated with etodolac. At an earlier time point, immediately after the end of ischemia, Tomlinson et al. (2004) did find increased COX-1 and COX-2 expression in ischemia-injured jejunum compared to control. Tomlinson et al. (2004) also found that both COX-1 and COX-2 were expressed in control uninjured equine tissue. In the current study, there was increased expression of COX-1 and trends towards increased expression of COX-2 in ischemia-injured
compared to control mucosa at 18 hours. The increase in expression in COX-1 and COX-2 is not surprising given the increase in neutrophilic infiltration into the mucosa in ischemia-injured mucosa seen in this study, and based on previous studies the elevation in COX-1 expression is likely to be due to increased numbers of neutrophils rather than increases in tissue expression, though this requires confirmation in the horse (Dupouy et al. 2006, Matsuyama et al. 2004). Although COX-1 is not typically upregulated in inflammatory conditions, there is evidence to suggest that expression of COX-1 is increased in the lamina propria mononuclear cells of gastric mucosa with increasing severity of mucosal ulceration (Bhandari et al. 2005). The neutrophilic infiltration in ischemia-injured mucosa compared to control mucosa may also account for the increased expression of PPARγ, and total P38 MAPK seen in saline treated horses. Furthermore, given that p38 MAPK is phosphorylated in response to chemotaxis, it is not surprising that the ratio of phospho-p38:total p38 increased in ischemia-injured mucosa of saline treated horses compared to control (Krump et al. 1997). There was a trend for meloxicam to reduce the upregulation of COX-1 expression in ischemia, and this was significant for COX-2. This may be due to alternate mechanisms of action of NSAIDs, since several NSAIDs are PPARγ ligands and activation of PPARγ is important for the transcriptional regulation of COX-2 (Chun et al. 2004, Tegeder et al. 2001). Peroxisome proliferator-activated receptor γ (PPARγ), a nuclear hormone receptor that functions as a transcription factor is known to be an important mediator of inflammatory gene transcription (Tegeder et al. 2001). Regulation of PPARγ expression in ischemia-reperfusion has not been well studied, however, PPARγ expression is down-regulated in alveolar macrophages of patients with allergic asthma (Kobayashi et al. 2005), suggesting
that expression may be responsive to change in inflammatory diseases. The role of PPARγ signaling in equine intestine deserves further attention, particularly given that meloxicam appears to inhibit the upregulation of its expression in ischemia. Expression of the mitogen activated protein kinases has also not been well studied in ischemia, even though some of the members of this group, ERK 1/2, JNK and p38MAPK are activated in response to ischemia and other stress and are critical for epithelial barrier function, stabilization of COX-2 mRNA, and inflammation. (Tegeder et al. 2001, Shifflett et al. 2004). In the equine jejunum, genes of 3 members of the mitogen activated protein kinases, Mapk 14, Mapk 1, Mapk 5 are down-regulated 7.45, 5.81, 3.59 –fold respectively immediately after the end of 2-hours of jejunal ischemia (unpublished data, Crisman, 2005) and in a rodent gastric ischemia-reperfusion injury model, Mapk 1 is down-regulated approximately 5-fold immediately after the end of ischemia, but is upregulated approximately 2-fold after 60 minutes of reperfusion (Naito et al. 2005). It is interesting that flunixin and especially meloxicam are able to reduce the upregulation of total p38 expression that occurs in ischemia and subsequent reperfusion. The fact that both of these NSAIDs also inhibit phosphorylation of p38 may point to alternative anti-inflammatory mechanism for the NSAIDs commonly used in the horse, as has been identified with other NSAIDs in experimental situations (Tegeder et al. 2001).

These data demonstrate that meloxicam may be an attractive alternative to flunixin meglumine for treatment of horses with colic, through its ability to provide adequate analgesia and beneficial effects on clinical parameters while permitting recovery of intestinal
barrier function. The results of this study also suggest that the effects of commonly used NSAIDs in the horse be evaluated for cyclooxygenase-independent mechanisms of action.

Acknowledgements

This study was funded by the American Quarter Horse Association. S.A. Brown was funded by the Merck-Meriel Summer Internship program at the College of Veterinary Medicine, North Carolina State University.

Footnotes


b Sigma, St Louis, MO, USA
c Agilent Technologies, Wilmington, DE, USA
d Waters Corporation, Milford, MA, USA
e WinNonlin, Version 4.0, Pharsight, Mountain View, CA, USA
f Biorad, Hercules, CA, USA
g Hybond ECL; Amersham Life Science, Birmingham, UK
h Santa Cruz Biotechnology, Inc, Santa Cruz, Ca, USA
i Abcam, Cambridge MA, USA
j Cell Signaling Technology Inc, Danvers, MA, USA
k Amersham, Piscataway, NJ, USA
l IP gel; Scanalytics, Fairfax, VA
References


Table 1: Behavioral Pain Scoring System. Scores from each category are added to give a total subjective pain score. NA = not applicable. Modified from Pritchett et al. (2003).

<table>
<thead>
<tr>
<th>Behavior category</th>
<th>Behavioral score to be assigned for each category</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Gross pain</td>
<td>None</td>
</tr>
<tr>
<td>Head position</td>
<td>Above withers</td>
</tr>
<tr>
<td>Ear position</td>
<td>Forward, frequent movement</td>
</tr>
<tr>
<td>Location</td>
<td>At door watching environment</td>
</tr>
<tr>
<td>Spontaneous locomotion</td>
<td>Moves freely</td>
</tr>
<tr>
<td>Response to another horse</td>
<td>Ears forward, head up, moves to door</td>
</tr>
<tr>
<td>Response to open door</td>
<td>Moves to door</td>
</tr>
<tr>
<td>Response to approach</td>
<td>Moves to observer, ears forward</td>
</tr>
<tr>
<td>Lifting feet</td>
<td>Freely when asked</td>
</tr>
</tbody>
</table>
Figure 1: Total pain scores for 3 treatment groups of 6 horses before 2-hours of jejunal ischemia, and 2 hours, 8 hours and 16 hours after the end of ischemia. Horses were treated with saline (S), flunixin meglumine (F), or meloxicam (M) perioperatively. * = significantly increased compared to pre-operative group at all time points. # = significantly greater to flunixin or meloxicam treatment at the same time point. P≤0.001
Figure 2: Mean (±SEM) Heart Rate (A) and respiratory rate (B) for three treatment groups of 6 horses before 2-hours of jejunal ischemia, and 2 hours, 8 hours and 16 hours after the end of ischemia. Horses were treated with saline (S), flunixin meglumine (F), or meloxicam (M) perioperatively. * = significantly greater than pre-operative values. # = significantly less than saline at the same time point. + = significantly less than flunixin at the same time point. P<0.05
Figure 3: Mean (± SEM) transepithelial electrical resistance (TER) in control and ischemia-injured equine jejunum 18 hours after the end of ischemia measured over a one-hour period after a one hour equilibration period on the Ussing chambers. Six horses in each of three treatment groups (saline, flunixin, meloxicam) * = significantly different to all other groups except ischemia-injured jejunum from horses treated with meloxicam. + = significantly different to all other groups except ischemia-injured jejunum from horses treated with saline. P<0.001
Figure 4: (A) Inulin flux across equine jejunum over a 1-hour period in the Ussing chamber. There was a trend P=0.07 for greater flux of inulin in ischemia-injured jejunum of horses treated with flunixin compared to horses treated with meloxicam or control jejunum of horses treated with saline. (B) LPS flux across equine jejunum over a 1-hour period in the Ussing chamber. * = significant (P=0.002) increase in LPS flux compared to all control jejunum and to ischemia-injured jejunum from horses treated with meloxicam P=0.375 compared to ischemia-injured tissue from horses treated with saline.
Figure 4C: Representative epifluorescent histological sections of control (A) and ischemia-injured (B) jejunum from horses treated with saline, 18-hours after the end of ischemia, and after 2-hours in the Ussing chamber with FITC-LPS applied to the luminal side of the tissue only. Note the increase in FITC staining in the epithelium, and submucosa of the ischemia-injured tissue. (x200)
Figure 5: Numbers of mucosal neutrophils at the end of 2-hours of ischemia (A), and 18-hours after the end of 2-hours of ischemia (B) in control and ischemia-injured equine jejunum from horses treated with saline, flunixin or meloxicam (n=6/group). * = significantly greater than control tissue. + = significantly greater than ischemia-injured mucosa with saline treatment. P<0.05
Figure 6: Mean percent of villus (±SEM) denuded of epithelial cells in control or ischemia-injured equine jejunum at the end of ischemia and 18 hours after the end of ischemia in horses treated with saline, flunixin or meloxicam (n=6). * = significantly greater than control jejunum (P<0.001). + significantly less than ischemia-injured flunixin treated jejunum, not different to meloxicam control (P<0.05).
Figure 7A: Mean (±SEM) villus height in control or ischemia-injured equine jejunum at the end of 2-hours of ischemia, or 18-hours after the end of 2-hours of ischemia. * = less than control jejunum at the same time point (P<0.001)
Figure 7B: Histological sections (H&E) of representative villi from (A) Control uninjured jejunum, (B) Ischemia-injured jejunum at the end of 2-hours of ischemia, and ischemia-injured jejunum 18 hours after the end of ischemia from horses treated with saline (C), flunixin (D) or meloxicam (E).
Table 2: Summary of noncompartmental pharmacokinetic parameters derived following IV administration of meloxicam (0.6 mg/kg) to 6 horses, and comparison with the existing equine data available.

Cp0 = maximum concentration; $AUC_{0-\infty}$ = area under the concentration-time curve; $AUMC_{0-\infty}$ = area under the first moment-time curve; $\lambda$ = slope of the terminal phase; $t1/2\lambda$ = half-life of terminal phase; Cl = systemic clearance; Vdarea = apparent volume of distribution

<table>
<thead>
<tr>
<th>Pharmacokinetic Variable</th>
<th>Mean (± SD)</th>
<th>Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cp0 (µg/mL)</td>
<td>9.78 ± 1.0</td>
<td>9.23 (Lees et al)</td>
</tr>
<tr>
<td>$AUC_{0-\infty}$ (hr*µg/mL)</td>
<td>33.44 ± 10.58</td>
<td>14.53 (Lees et al)</td>
</tr>
<tr>
<td>$AUMC_{0-\infty}$ (hr<em>hr</em>µg/mL)</td>
<td>151.61 ± 85.39</td>
<td></td>
</tr>
<tr>
<td>$\lambda$ (hr -1)</td>
<td>0.18 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>$t1/2\lambda$ (hr)</td>
<td>4.07 ± 1.07</td>
<td>2.7 (Lees et al) 5.15 (Toutain &amp; Cester) 8.54 (Toutain et al)</td>
</tr>
<tr>
<td>Clearance (mL/kg/min)</td>
<td>0.33 ± 0.14</td>
<td>0.68 (Lees et al) 1.35 (Toutain &amp; Cester) 0.58 (Toutain et al)</td>
</tr>
<tr>
<td>Vdarea (L/kg)</td>
<td>0.11 ± 0.04</td>
<td>0.16 (Lees et al)</td>
</tr>
</tbody>
</table>
Figure 8: Plasma concentrations of meloxicam in horses treated with 0.6 mg/kg meloxicam intravenously immediately prior to induction of general anesthesia and 2-hours of segmental jejunal ischemia, then 18 hours of recovery. (Mean±SD) n=6.
Figure 9: Western Blot analysis of control (C) and ischemia-injured (I) mucosa from 3 representative horses (1,2,3) in each drug treatment group (Saline, Flunixin, Meloxicam) 18 hours after the end of ischemia, probed for COX-1, COX-2, PPARγ, Total P38, phosphorylated P38 and B-actin (loading control)
Figure 10: Densitometry analysis for COX-1 (A,B), COX-2 (C,D), PPARγ (E,F), Total P38 (G,H) and the ratio of phospho-P38:total P38 (I,J) for control and ischemia-injured tissues from 3 representative horses in each drug treatment group 18-hours after the end of ischemia. (A,C,E,G) represent mean densitometry units (± SEM), (B,D,F,H) represent percent change (± SEM) in densitometry between control and ischemia-injured tissue within the same horse. (I,J) are ratios of phosphoP38:total P38 derived from densitometry. (I) represents the change in ratio (± SEM) between tissues from each of the three drug treatment groups, referenced to control tissue from saline treated horses. (J) represents the change in this ratio (± SEM) in ischemic compared to control tissue for the three drug treatment groups. P<0.05.

**COX-1** (A,B) * significant increase ischemia-injured vs control tissue. **COX-2** (C,D) Trend (P=0.08) for increased expression in ischemia-injured vs control tissue * significant difference to meloxicam group. **PPARγ** (E,F) * significant difference to all other groups. # significant difference to control tissue from saline treated horses. + significantly less than saline or flunixin. **Total P38** (G,H) * significant difference to all other groups. + significant difference to control tissue from meloxicam and flunixin treated horses. **Ratio phospho-P38:total P38** (I,J) * significant difference between groups.
Arbitrary Densitometry Units

Control Ischemic Control Ischemic Control Ischemic

Meloxicam Flunixin Saline

% increase

0 20 40 60 80 100 120 140 160 180

Saline Flunixin Meloxicam

Change in ratio

0 2 4 6 8 10 12 14 16

Control Ischemic Control Ischemic Control Ischemic

Saline Flunixin Meloxicam

Ratio phospho-P38:Total P38

0 2 4 6 8 10 12 14 16

Control Ischemic Control Ischemic Control Ischemic

Saline Flunixin Meloxicam