

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA

UMI[®]
800-521-0600

NOTE TO USERS

**Page(s) missing in number only; text follows.
Microfilmed as received.**

**34,57,60,82,113, 116,
140,156,158,164,168**

This reproduction is the best copy available.

UMI

ACUTE STRESS-INDUCED PATHOPHYSIOLOGY OF INTESTINAL EPITHELIUM
IN
STRESS SUSCEPTIBLE WISTAR KYOTO RATS

By
PAUL RUSSELL SAUNDERS, B.Sc.

A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree
Doctor of Philosophy in Medical Science

McMaster University

© Copyright by Paul Saunders, April 1998

10/10/10

STRESS-INDUCED PATHOPHYSIOLOGY OF INTESTINAL EPITHELIUM

DOCTOR of PHILOSOPHY (1998)
(Medical Science)

McMaster University
Hamilton, Ontario

TITLE: Acute Stress-Induced Pathophysiology of Intestinal Epithelium in
Stress Susceptible Wistar Kyoto Rats

AUTHOR: Paul Russell Saunders, B.Sc. (McMaster University)

SUPERVISOR: Dr. Mary H. Perdue (Professor)

NUMBER OF PAGES: xix, 177

To me & Lil' Paul
(despite our best efforts I got it done),
and dedicated to the memory of Caleb Saunders

ABSTRACT

The gastrointestinal tract is particularly sensitive to stress. Stress-induced gastric ulceration and stress-induced alterations in motility have been examined. The purpose of my studies was to define stress-induced changes in the intestinal epithelium and the mechanisms involved in the epithelial responses.

Wistar Kyoto rats were stressed by restraint. Jejunal and colonic tissues from stressed or control rats were removed and parameters of epithelial physiology were studied in Ussing chambers. Acute stress caused a significant increase in intestinal chloride ion secretion. In addition, tissues from stressed rats demonstrated impaired responses to neural activation. Compared to controls tissues from stressed rats were also found to have increased permeability to ions, and small inert probes, and increased permeability to a macromolecular protein. In spite of these functional abnormalities, the mucosa showed no signs of damage. Our next series of experiments showed that peripheral cholinergic nerves and corticotropin-releasing factor were responsible for mediating these epithelial responses to acute stress. Pretreatment with atropine (jejunum) or a corticotropin-releasing factor antagonist (colon) prevented the stress-induced pathophysiology, while administering corticotropin-releasing factor mimicked the colonic responses. These studies also showed that the Wistar Kyoto strain of rats developed more extreme intestinal abnormalities to stress than the parental

Wistar strain, most likely due to the fact that Wistar Kyoto rats have less cholinesterase activity.

In summary, my studies showed that stress impaired epithelial function along the intestinal tract. We speculate that in susceptible individuals, acute stress can cause the epithelial barrier to become leaky allowing greater uptake of small proinflammatory molecules (bacterial products) as well as larger macromolecules (antigens) from the lumen. Subsequent stimulation of immunocytes may initiate or exacerbate inflammation.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. M.H. Perdue. She provided a wonderful laboratory to work in and innumerable opportunities to learn. I cannot possibly list all the invaluable lessons learned, the most notable ones permitted me to gain insight into 'what it takes' to survive in research, thanks Mary.

I would like to thank my committee members, Drs. J. Bienenstock and D. deCatanzaro, for their support, providing the gentle guidance steering me in the best direction possible but still allowing me to participate and make worthwhile decisions throughout my project. Thank you John and Denys.

I would like to thank the entire IDRП for their kindness, support, and friendship. A special thanks to the faculty who befriended me and in their own fashion provided a wealth of knowledge and experience I was able to glean from. Thanks to Steve Collins, P.K. "Chari" Rangachari, Gervais Tougas, Michael Blennerhassett, Andrzej Stanisз, Sheila Crowe, Carlos Barajas-Lopez and I will never forget the special friendship and support of Jean Marshall. A special thanks to my good friends Lu Wang, Cory Hogaboam, Bruce Vallance, Scott Simpson, Kristian "mud butt" Davis, also to Al, Giovanni, Nick, Jennifer, Milan, Rene, Brian, Frank, Gert, Ruth, Tom, Fern, Pete, Ronan, Jane Ann, Diane, Michelle, Mike and of course Rosa Espinoza-Luna.

Thanks to my comrades in the trenches, all of whom made my time memorable and enjoyable. Thank you Ula, Veljko "schticky" Djurić, Pam, Ping-Chang, Jun, Galina, Mehri, Dihan, Nico, Derrick, Graeme, Barbara, to my fellow inmate Cecilia Berin and last but definitely not least the incomparable Michelle "supertech" Benjamin (who saved my bacon more times than I care to mention). A thanks to all my lab friends in Amsterdam (Rajash, Judith, Luda and Anya), special thanks to Amanda Kiliaan (with Frank-Eric and Lil' Loedweg), Pieter Bijlsma, Hetty Bouritius and Jack Groot. I shared some special times with these people.

Words can't express the thanks I bestow on my close personal friends which during the dark days still made it fun to come to work. To the lads "Eddy" Todd Prior and Pierre "Pappy" Betti, thanks for everything (since last we met look what I've got done). To Derek "Lucky Leprechaun" McKay you are one of a kind, thank God for that. To Todd Braciak who told me before I started it would be the best of times, **it would be the worst of times**, he was right, thanks man (although I think stress affected me more than 5%).

To the players of the Natural Killers softball teams and the Green Genes football teams, thank you for some of the most enjoyable summers of my life. Special thanks to Bomber, Wally & Ine, Vincent & Isabelle with Lil' Lucie, Pip "pepper" & Darren, Tina, Phillsy, Carl, Gerry, Frankie, 'Smiebs', Dereck C., Jimmy and Craig.

Thanks to Ken Beal, not just a friend but a fine mentor too, and Kathy Hancock for keeping me relatively sane for the duration.

Finally, a heartfelt thanks to my family, the only group of people that could make my friends look civil. Thanks to my mom, Janet, and my dad, Rod, for the love, support and encouragement you gave (which of course in this household translates into teasing, nagging and extorting). To my brother, David, and my sister, Mary, thank you for the ceaseless critiquing and undaunting harassment, I love you two too. To Caleb for your unconditional love and unique perspective on life, I love you and miss you dearly (the bestus babies).

Paul

TABLE of CONTENTS

INTRODUCTION	1
1.1 GASTROINTESTINAL TRACT	3
1.1.1 General Function	3
1.1.2 General Structure	3
1.1.3 Epithelium	4
1.1.3.1 Composition	4
1.1.3.2 Function	8
1.1.3.3 Tight Junctions	9
1.1.3.4 Macromolecular Transport	15
1.1.4 Enteric Nervous System	16
1.2 STRESS	21
1.2.1 Historical Perspective	21
1.2.2 Definition of Stress	23
1.2.3 Central Mediation of Stress	25
1.2.4 Stress & The Gastrointestinal Tract	27
1.3 STUDY PLAN	31

Acute stressors stimulate ion secretion and increase epithelial permeability in rat intestine 33

ABSTRACT 35

 2.1 **INTRODUCTION** 35

 2.2 **METHODS** 36

 2.2.1 **Animal Model** 36

 2.2.2 **Ussing Chamber Studies** 37

 2.2.3 **Histology** 39

 2.2.4 **Statistics** 40

 2.3 **RESULTS** 40

 2.3.1 **Corticosterone** 40

 2.3.2 **Ion Transport** 41

 2.3.3 **Permeability** 46

 2.3.4 **Histology** 47

 2.4 **DISCUSSION** 47

 2.5 **REFERENCES** 54

Cholinergic nerves mediate stress-induced intestinal transport abnormalities in Wistar Kyoto rats.	59
ABSTRACT	61
3.1 INTRODUCTION	61
3.2 METHODS	63
3.2.1 Animals	63
3.2.2 Ussing Chamber Studies	64
3.2.3 Permeability	64
3.2.4 Cholinergic Receptor Antagonism	65
3.2.5 Cholinesterase Activity	66
3.2.6 Statistics	66
3.3 RESULTS	66
3.3.1 Intestinal Parameters in Control and Stressed Wistar and Wistar Kyoto Rats	66
3.3.2 Effect of Cholinergic Antagonism on Stress-Induced Intestinal Pathophysiology	68
3.4 DISCUSSION	71
3.5 REFERENCES	77

Stress stimulates transepithelial macromolecular uptake in rat jejunum.	81
ABSTRACT	83
4.1 INTRODUCTION	83
4.2 METHODS	86
4.2.1 Animals	86
4.2.2 Ussing Chamber Studies	87
4.2.3 Permeability	88
4.2.4 Protein Transport	88
4.2.5 Electron Microscopy	89
4.2.6 Statistical Analysis	90
4.3 RESULTS	90
4.3.1 Tissue Conductance and Permeability to Cr-EDTA	90
4.3.2 Protein Transport	91
4.3.3 Electron Microscopy	91
4.3.3.1 Transcellular Pathway	91
4.3.3.2 Paracellular Pathway	94
4.4 DISCUSSION	95
4.5 REFERENCES	109

Stress-induced epithelial pathophysiology in rat colon: role of corticotropin-releasing factor	115
ABSTRACT	117
5.1 INTRODUCTION	118
5.2 METHODS	120
5.2.1 Animal Model	120
5.2.2 Drug Administration	121
5.2.2.1 Atropine	121
5.2.2.2 CRF/CRF Antagonist	121
5.2.3 Ussing chamber studies	121
5.2.4 Permeability	122
5.2.5 Protein transport	122
5.2.6 Electron microscopy	123
5.2.7 Statistical Analysis	124
5.3 RESULTS	124
5.3.1 Intestinal Parameters in Control and Stressed Wistar and Wistar Kyoto Rats	124
5.3.2 Electron microscopy	125
5.3.3 Mediators of the Stress Response	130
5.3.3.1 Acetylcholine	130
5.3.3.2 CRF	130

5.4	DISCUSSION	133
5.5	REFERENCES	136
DISCUSSION		139
6.1	SUMMARY	141
6.1.1	Objective 1: To Define Stress-Induced Pathophysiology of Intestinal Epithelium.	141
6.1.2	Objective 2: To Explore Mechanisms Responsible for Stress- Induced Pathophysiology of Intestinal Epithelium	143
6.2	CLINICAL SIGNIFICANCE & SPECULATIONS	147
6.3	FUTURE DIRECTIONS	151
6.3.1	Further Mechanisms Involved in the Stress Responses	152
6.3.2	Stress Mediated Intestinal Inflammation Models	153
6.4	OVERALL SIGNIFICANCE	155

APPENDIX A	157
A.1 EXTENDED IDEAS REGARDING STRESS	159
A.1.1 General Adaptation Theory	159
A.1.2 Defining Stress (Inducer of a Prepathological State)	159
A.1.3 Defining My Stress Model	161
APPENDIX B	163
B.1 TIME COURSE DATA	165
REFERENCES	169

LIST of TABLES

TABLE 1.1 Putative Enteric Neurotransmitters	18
TABLE 2.1 Na ⁺ and Cl ⁻ Fluxes in Jejunal Tissues from Control and CRS Rats	46
TABLE 2.2 Morphometric Measurements of Jejunal Tissues	47
TABLE 3.1 Transport Parameters in Jejunum from Wistar and Wistar Kyoto Rats	67
TABLE 4.1 Conductance Values for Jejunal Tissues	91
TABLE 5.1 Electrophysiological Parameters in Colonic Mucosa	125
TABLE 5.2 Mediators of the Colonic Mucosal Stress Responses	132

LIST of FIGURES

Introduction

Figure 1.1 General structure of the intestinal tract	7
Figure 1.2 Epithelial cell phenotypes	11
Figure 1.3 Ion transport across epithelial cells	12
Figure 1.4 Terminal bar	14
Figure 1.5 Autonomic innervation of the GI tract	22
Figure 1.6 Central mediation of GI stress responses	29

Chapter 2

Figure 2.1 Baseline short-circuit current	43
Figure 2.2 Short-circuit current responses to stimulation	45
Figure 2.3 Permeability of intestinal epithelium	49

Chapter 3

Figure 3.1 Cholinergic measures in Wistar and Wistar Kyoto rats	69
Figure 3.2 Baseline short-circuit current in Wistar Kyoto rats	70
Figure 3.3 Intestinal permeability in Wistar Kyoto rats	73

Chapter 4

Figure 4.1 Molecular fluxes of intestinal epithelium	93
Figure 4.2 Electron photomicrographs of intracellular HRP	97
Figure 4.3 Electron photomicrographs of paracellular HRP	99
Figure 4.4 Quantification of endosomal HRP	101
Figure 4.5 Electron photomicrographs of lamina propria	103

Chapter 5

Figure 5.1 Effect of stress on colonic transport in Wistar and Wistar Kyoto rats	127
Figure 5.2 Electron photomicrographs of intestinal tissues	129
Figure 5.3 Quantification of endosomal HRP in colonic epithelium	131

Discussion

Figure 6.1 Schema of mechanisms mediating the stress-induced intestinal epithelial pathophysiology	149
---	-----

Appendix B

Figure B.1 Time course of intestinal epithelial stress responses	167
--	-----

FORMAT & ORGANIZATION OF THESIS

This thesis is organized in the “Open-faced” sandwich format, approved by McMaster University. This thesis is comprised of 6 chapters. The first chapter is a general introduction and is followed by two chapters, chapters 2 and 3, which are published manuscripts in a peer reviewed scientific journal. Chapter 4 is a manuscript submitted for publication and following this chapter 5 is a manuscript being prepared for submission to a scientific journal. Chapter 6 is a general discussion highlighting the overall significant contributions and ramifications for the work presented in the four manuscripts. Appendices have been included, appendix B provides key preliminary data not presented in the four manuscripts.

Contributions to Multi-Authored Papers

Chapter 2: Acute stressors stimulate ion secretion and increase epithelial permeability in rat intestine. 1994.

Authors: P.R. Saunders, U. Kosecka, D.M. McKay and M.H. Perdue.

Journal: American Journal of Physiology, 267: G794-G799.

Comment: The experiments were conducted and data collected by the author P.R. Saunders with the aid of Drs. U. Kosecka and D.M. McKay under the supervision of Dr. M.H. Perdue.

Chapter 3: Cholinergic nerves mediate stress-induced intestinal transport abnormalities in Wistar Kyoto rats. 1997.

Authors: P.R. Saunders, N.P.M. Hanssen and M.H. Perdue.

Journal: American Journal of Physiology, 273: G486-G490.

Comment: The experiments were conducted and data collected by the author P.R. Saunders with the aid of N.P.M. Hanssen (completing requirements for an undergraduate thesis with the guidance of P.R. Saunders) under the supervision of Dr. M.H. Perdue.

Chapter 4: Stress stimulates transepithelial macromolecular uptake in rat jejunum. 1998.

Authors: A.J. Kiliaan, P.R. Saunders, P.B. Bijlsma, C. Berin, J.A. Taminiau, J.A. Groot and M.H. Perdue.

Journal: American Journal of Physiology, submitted.

Comment: The experiments were conducted by P.R. Saunders; the flux data was collected and analysed by P.R. Saunders. The electron microscopic evidence was prepared and analysed predominantly by Dr. A.J. Kiliaan with additional data provided by C. Berin. P.B. Bijlsma was instrumental in the initial experimental design. Supervision for this project was provided by Drs. M.H. Perdue, J.A. Groot and J.A. Taminiau.

Chapter 5: Stress-induced epithelial pathophysiology in rat colon: role of corticotropin-releasing factor. 1998.

Authors: P.R. Saunders, N.P.M. Hanssen, P-C. Yang, D. Yates, J.A. Groot and M.H. Perdue.

Journal: in preparation

Comment: The experiments were conducted and data collected by the author P.R. Saunders with the aid of N.P.M. Hanssen and D. Yates (completing requirements for an undergraduate thesis and a summer lab placement, both with guidance from P.R. Saunders) except for the electron microscopic data which was prepared and analysed by Dr. P-C. Yang. Supervision for this project was provided by Drs. M.H. Perdue and J.A. Groot.

Chapter 1

INTRODUCTION

Belief Creates Biology

Norman Cousins

Yesterday's stress is past tense

Sherrie Weaver

INTRODUCTION

1.1 GASTROINTESTINAL TRACT

1.1.1 General Function

The gastrointestinal tract runs from the mouth to the anus, with each region serving specific functions in digestion, absorption of nutrients and expulsion of waste. The mouth begins the digestion of food by breaking down foodstuff mechanically, with chewing, and chemically, with salivary secretions. The partially broken down food is swallowed and enters the stomach where acid and enzymes are secreted to further break down the contents. This churned up mixture is forced into the duodenum, the first segment of the small intestine. Secretions from the liver and pancreas enter the lumen, neutralizing the stomach's acid and aiding the digestion of sugars, proteins and fats for manageable absorption. The mixture moves along the small intestine where the absorption of nutrients occurs predominantly in the jejunum and to a lesser extent in the ileum. The luminal contents are passed through the cecum to the large intestine where the last of the water and salts are recaptured and the stools solidified in preparation for defecation via the rectum.

1.1.2 General Structure

Each region of the intestinal tract is composed of the same classical mucosal structure. The thin outer layer is the serosa which surrounds the intestinal tissue. Next is the muscularis

layer composed of two smooth muscle layers the longitudinal smooth muscle and the circular smooth muscle, perpendicular to one another. The myenteric nerve plexus is located between the two layers and coordinates their movement; fibres from the myenteric plexus project to the central nervous system, via autonomic nerves and to the submucosal nerve plexus. The submucosa is made up of connective tissue with elastic fibres and contains the submucosal nerve plexus and the larger blood vessels and lymphatics of the intestine. Nerves of the submucosal plexus regulate the absorptive and secretory functions of the mucosa, and like the myenteric plexus also have connections with the central nervous system.

The mucosa is the innermost layer and contains many different cell types including parenchymal cells, immunocytes, endothelial cells of the vascular/lymphatic networks and epithelial cells. The major parenchymal cells are the fibroblasts which form the scaffolding supporting the villi and crypts. Also present in the mucosa are a number of immunocytes, including lymphocytes, macrophages, neutrophils, eosinophils and mast cells, which are involved in protecting the organism from invading viruses, bacteria and parasites. Lining the entire mucosa is a single layer of contiguous epithelial cells. See figure 1.1.

1.1.3 Epithelium

1.1.3.1 Composition

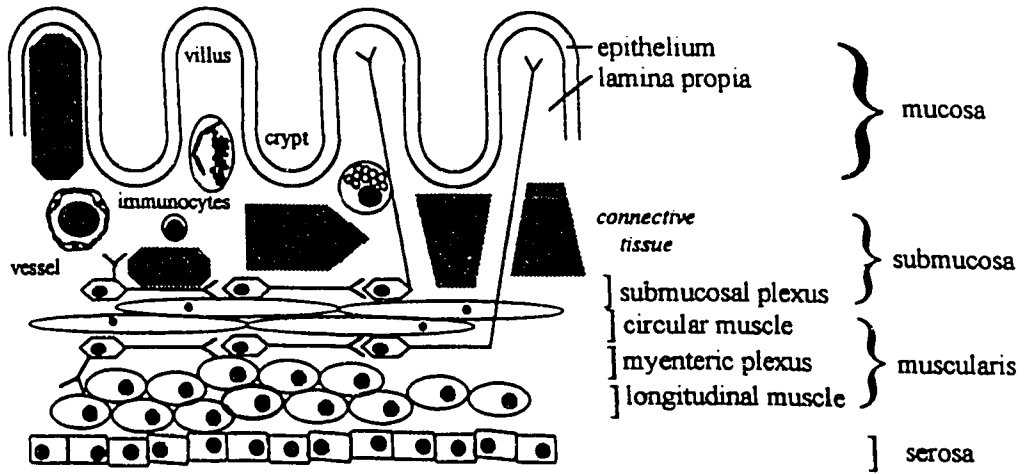
Creating the barrier between the external and internal environments of the body is a single layer of epithelial cells lining the entire luminal boundary of the gastrointestinal tract. By far the most representative cell type composing the epithelial surface is the transporting enterocyte. In the small intestine, stem cells near the base of the crypts give rise to enterocytes

that are primarily secretory in the crypt and become absorptive as they migrate and mature up the villus. The colon has crypts only, although colonic surface epithelial cells generally behave like the absorptive villus epithelial cells of the small intestine. When cells reach the tips of the villi they are sloughed into the lumen or resorbed and broken down by macrophages in the lamina propria; the average life span of an enterocyte is 4-7 days. M cells are highly specialized enterocytes in the epithelium, covering Peyer's patches. Relative to other individual epithelial cells, M cells have been shown to absorb significantly more particulate luminal material (Madara & Trier, 1994).

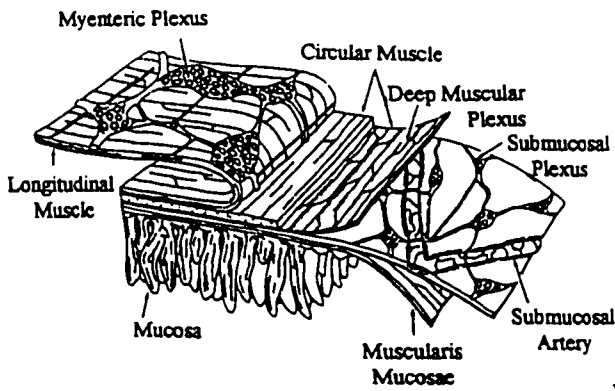
Another key cell in the epithelium is the goblet cell. Goblet cells produce and secrete mucus into the intestinal lumen. The mucus helps to lubricate the luminal contents as well as to create a very thin, but none the less very important barrier, called the unstirred layer, that protects the epithelium. Mucus limits direct exposure of the epithelium to toxins and bacteria present in the lumen, and aids in the passage of abrasive and potentially damaging material, as the luminal contents are forced through the intestine. Enteroendocrine cells are also located throughout the intestinal epithelium. These endocrine cells are characterized by their granular structure and their composition of intestinal hormones; some of the hormones packaged in enteroendocrine cells include serotonin, cholecystokinin, somatostatin and substance P. The hormones released at the basal membrane may affect local cells in a paracrine fashion and/or may also be taken up in the blood, affecting target cells at remote regions in the classic endocrine fashion. Another key epithelial cell type is the Paneth cell. These cells are found at the base of the crypts. They are thought to be protective cells since they possess vacuoles containing defensins, highly toxic and degradative enzymes most likely used to kill invading

Figure 1.1 General structure of the intestinal tract. A) General schema depicting the characteristic structures observed throughout the entire thickness of intestinal tissue, representative of the small intestine (modified from Madara, 1995). B) Schema demonstrating the complexity of enteric plexuses alone (modified from Furness & Bornstein, 1995). C) Schema demonstrating the complexity of mucosal vasculature alone (modified from Crissinger & Granger, 1995).

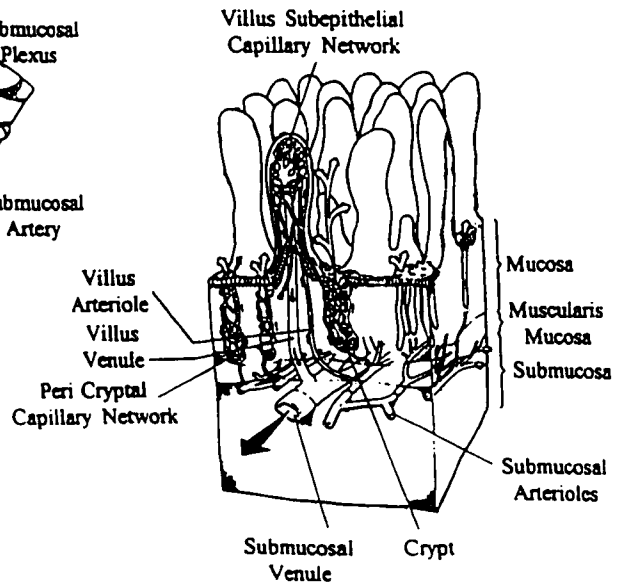
A



B



C



bacteria (Madara & Trier, 1994). Embedded in the epithelial layer, above the basement membrane but not directly exposed to the lumen, is a type of immunocyte known as the intraepithelial lymphocyte. Intraepithelial lymphocytes are a unique population of lymphocytes compared to lamina propria and systemic lymphocytes. They demonstrate T cell phenotypes (although some intraepithelial lymphocytes are thymus dependent while another subpopulation are thymus independent), almost exclusively CD8⁺, and show cytotoxic T-lymphocyte activity. Their exact role is unknown although under certain circumstances they demonstrate natural killer-like activity. There is speculation that intraepithelial lymphocytes may be involved in the complex regulation of oral tolerance (Shanahan, 1994). See figure 1.2.

1.1.3.2 Function

The primary purpose of the gastrointestinal tract is to absorb essential nutrients, vitamins and minerals from the lumen into the blood stream for distribution throughout the body. The epithelium is critical to all transport processes. It is generally accepted that villus epithelial cells are the absorptive cells while crypt epithelial cells are secretory cells (Kaunitz et al., 1995). Secretion of water is an integral part of digestion as it aids in the loosening and dissolving of luminal contents allowing easier access of nutrients to absorptive cells. In addition, water secretion helps wash antigens and other noxious material away from the epithelial apical membrane. The epithelium accomplishes both absorption and secretion by creating electrochemical gradients across cellular membranes. The Na⁺, K⁺ ATPase pump, in the basolateral membrane, provides the electrochemical gradients necessary for epithelial function. The expulsion of 3 sodium ions for 2 potassium ions with the consumption of one ATP molecule creates an electrical gradient, with the cell being more negative than the

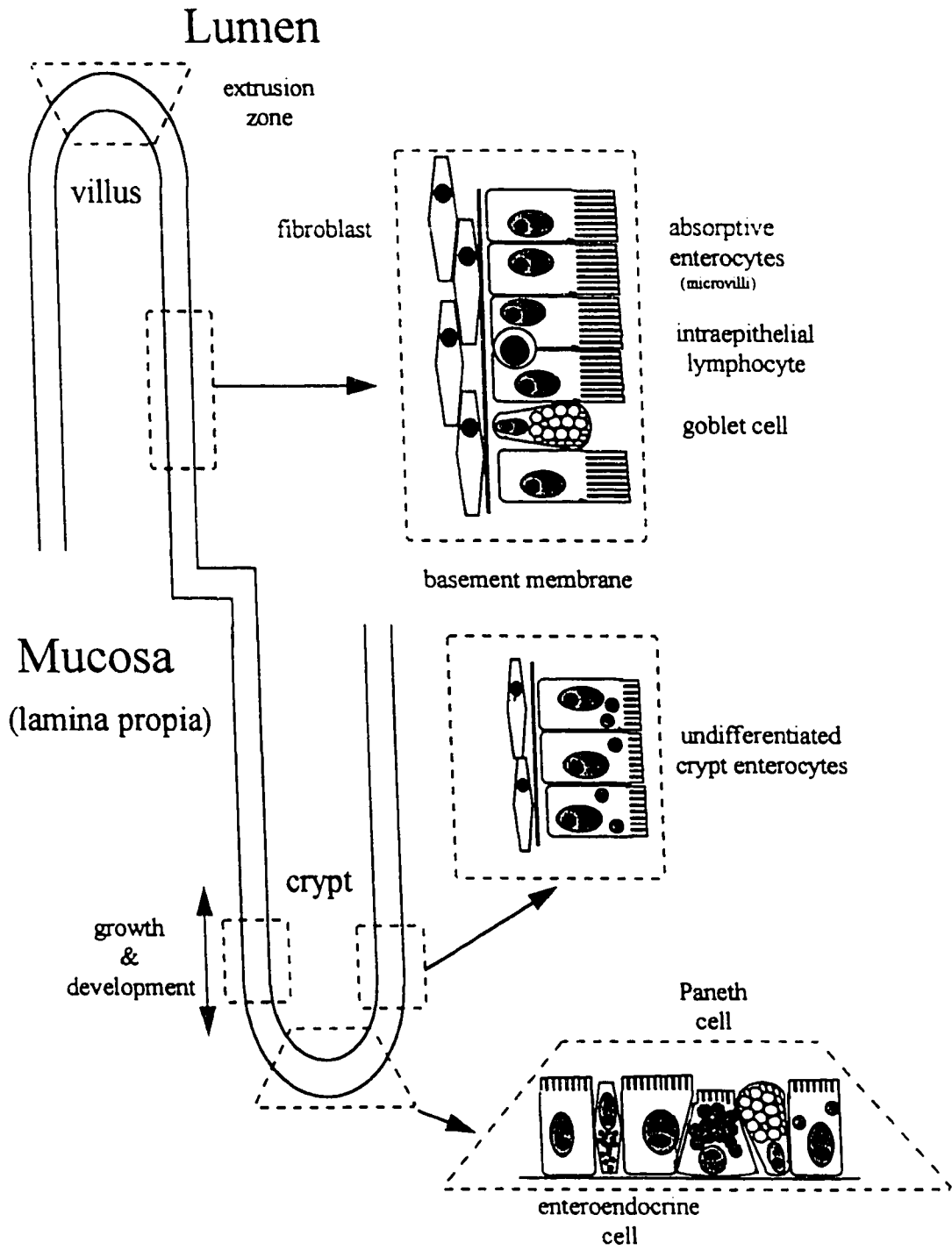
external environment of the serosa, as well as a specific concentration gradient favouring sodium entering the cell. The absorptive villus cell has cotransporters on the apical membrane allowing glucose, amino acids, peptides, minerals, vitamins, bile salts and fats to be absorbed along with sodium (Kaunitz et al., 1995). The secretory crypt epithelial cells use the same electrochemical gradient created by the basolateral Na^+ , K^+ ATPase pump to secrete water. The basolateral Na^+ , K^+ , Cl^- cotransporter allows for increased intracellular chloride ion concentrations despite the fact that the chloride transport is against the electrical gradient of the cell. When signalled, chloride ion channels in the apical membrane open, allowing chloride to flow into the lumen down its electrical chemical gradient. Sodium ions follow passively, between the epithelial cells (as opposed to through the cells) to counter the negatively charged chloride ions. The ion movement creates an osmotic gradient and water also moves passively from the cell and the mucosa into the lumen of the intestine. In addition, bicarbonate is also secreted by the epithelium (Cooke & Reddix, 1994; Kaunitz et al., 1995).

During absorption, an osmotic gradient is also being created in the villus epithelium as ions and neutral molecules are absorbed from the lumen. During this process, water also crosses the epithelium passively between the cells resulting in additional nutrients being absorbed passively by solvent drag (Madara, 1995). Thus, most of the water that was secreted by the body is recaptured, preventing the devastating problems of dehydration. See figure 1.3.

1.1.3.3 Tight Junctions

Just basal to the apical membrane, circumventing every epithelial cell, is a protein structure called the terminal bar which is intimately connected to the cytoskeleton. The terminal bar consists of tight junctions, adherens junctions and desmosomes. Adherens

Figure 1.2 Epithelial cell phenotypes. Schema of the different phenotypes of cells observed making up the epithelium along the entire villus/crypt axis. The villus epithelium is predominantly composed of absorptive enterocytes, with their longer microvilli, interspersed with the mucus-producing goblet cells. The occasional intraepithelial lymphocyte, squeezed between the bases of enterocytes, will be observed just above the basement membrane, created by the adjacent fibroblasts. The crypt is composed of less differentiated secretory enterocytes, and sometimes observed at the base of the crypts are the unusual and specialized Paneth cells along with enteroendocrine cells (modified from Madara & Trier, 1994; Madara, 1995).



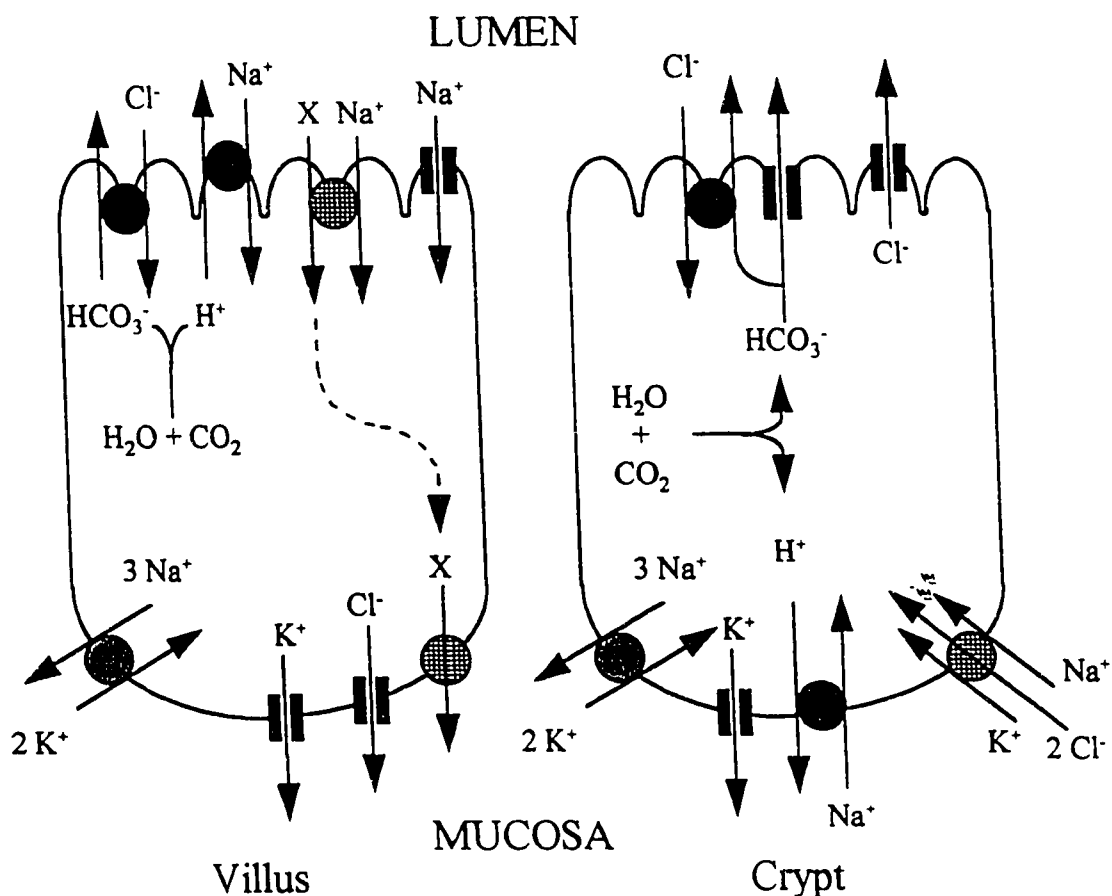


Figure 1.3 Ion transport across epithelial cells. Schema of the ion transport across villus and crypt enterocytes. The $\text{Na}^+\text{K}^+\text{ATPase}$ pump (shaded circles) creates a sodium gradient which is the driving force for ion transport in both the absorptive villus enterocyte and the secretory crypt enterocyte. Sodium cotransporters (checkered circle) in the villus enterocyte allows a number of vital nutrients, X, to be absorbed from the lumen. Such nutrients include glucose, amino acids, peptides, minerals, vitamins, bile salts and fats. Sodium/hydrogen and chloride/bicarbonate exchangers (solid circles) allow for sodium and chloride to be reabsorbed; water follows the osmotic gradients created by the ions and nutrients. The $\text{Na}^+\text{K}^+\text{Cl}^-$ cotransporter (checkered circle) on the basal membrane supplies the crypt enterocyte with chloride which will be secreted into the lumen via a chloride channel (rectangular box). The bicarbonate anion may also be secreted via a bicarbonate channel or a chloride/bicarbonate exchanger (solid circle). Water is secreted following the ionic gradients. These are the general patterns of ion movements across epithelial cells. Note: there are regional and species differences for the exact transport of ions across specific enterocytes (modified from Kaunitz et al., 1995).

junctions and desmosomes seem to be very important in maintaining epithelial cells as a single layer of cells, while tight junctions maintain the barrier between the lumen and the mucosa (Madara & Trier, 1994). See figure 1.4.

Tight junctions were once thought to be completely impermeable, since it was observed that tracers (eg. lanthanum hydroxide) added to the basal side of epithelium diffused up the lateral space to the tight junctions where movement was halted. No evidence of the tracer could be found in the lumen on the apical side of the cells (Cereijido, 1991). Epithelial tissues used in early studies normally had very high resistances. Efforts eventually revealed that epithelial sheets from different sites, and species, have different resistances. Intestinal epithelium has a relatively low resistance and is considered a “leaky” epithelium. The leaky epithelial tissues permit ions and small molecules (in particular water) to pass through the tight junctions and cross the epithelium via the paracellular space (Fromter, 1972; Diamond, 1974). Frizzell and Schultz (1972), utilizing radiolabelled ions, diffusion potentials, and ion replacement buffers, indicated that the paracellular movement of sodium, chloride and potassium ions accounted for 82% of the total tissue conductance of rabbit ileum. A compelling hypothesis (Marcial et al., 1984) postulated that guinea pig crypt epithelium is much leakier than villus epithelium, with the crypt accounting for over 70% of the overall conductance measured across the ileum. This idea is based on mathematical modelling of the two epithelial compartments. Why villus epithelial cells have tighter junctions than crypt epithelial cells is unknown. However, villus epithelial cells have greater exposure to luminal contents and may need greater protection in preventing noxious material from bypassing the epithelium and entering the mucosa. Also, there may be greater regulation of the permeability

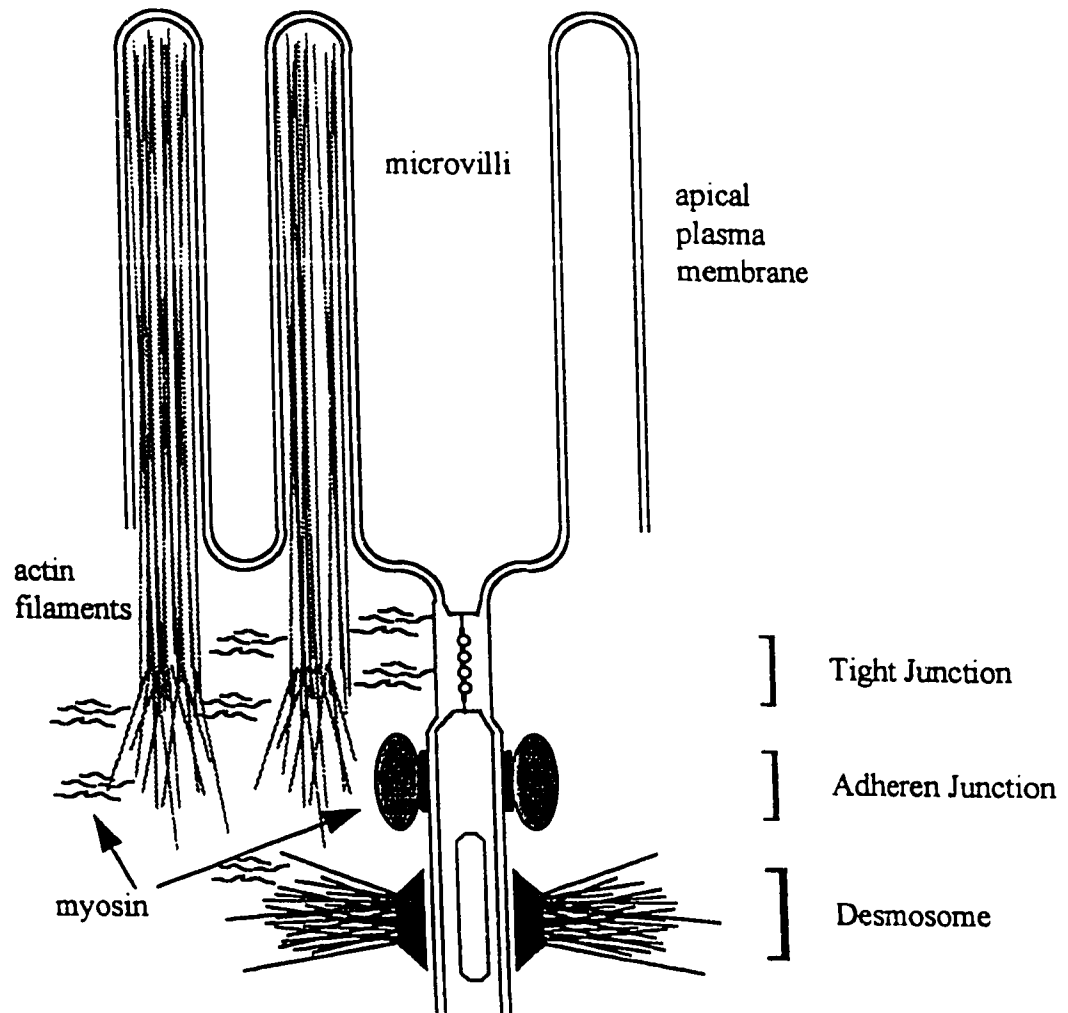


Figure 1.4 Terminal bar structure. The terminal bar is composed of tight junctions, adheren junctions, and desmosomes. Adheren junctions and desmosomes are involved in maintaining the integrity of the epithelial monolayer, while tight junctions are the paracellular barrier between the lumen and the intestinal mucosa. All three components of the terminal bar are intimately connected to the epithelial cytoskeleton (modified from Madara, 1995).

of villus epithelial cells allowing more solute and water to be absorbed (by solvent drag) only when active absorption is taking place. There is evidence that luminal glucose can increase tissue conductance and dilate the epithelial tight junction (Madara & Trier, 1994). As discussed earlier, the crypt cells actively secrete chloride ions (and bicarbonate), the driving force for secretion of sodium and water. Leakier tight junctions of crypt epithelia may result in a more rapid response facilitating flow of water into the lumen.

The resistance of tight junctions is not a static property but can be regulated. The resistance of epithelial tight junctions is altered by luminal nutrients, specifically glucose and the amino acid alanine. Luminal nutrients caused not only a decrease in epithelial resistance but also dilations in the tight junctions (Atisook & Madara, 1991; Sadowski & Meddings, 1993). Cytokines also may affect epithelial permeability. For example, interferon- γ decreased resistance across the T₈₄ human colonic epithelial cell line (Adams et al., 1993). Bacterial products such as clostridium toxin B or cholera toxin are known to affect tight junctions, decreasing transepithelial resistance of Caco2 (another colonic human epithelial cell line) and T₈₄ cells as well as rabbit ileum (Canil et al., 1993; Hecht et al., 1992; Fasano et al., 1991). Whether the epithelial responses are a defence reaction by the cells to aid in the washing away of noxious pathogens and substances, or whether they actually facilitate infection of the host by the bacteria, is not known. It is interesting to note that calcium not only increases chloride secretion but also increases tight junctional permeability in epithelial cells.

1.1.3.4 Macromolecular Transport

Macromolecules also cross the epithelial barrier, although most (>95%) are normally prevented from doing so. Specific macromolecules are required for survival, especially in

immature individuals, and there are specific receptor-mediated uptake mechanisms for molecules such as growth factors and antibodies. Receptors residing on the apical membrane bind macromolecules that are then endocytosed via clathrin coated pits. Molecules bound to their receptors are protected from the normal degradation by intracellular lysosomal enzymes, and are transported to the basolateral membrane and released into the circulation. Nonspecific endosomal uptake also occurs. Therefore, any macromolecules in the lumen may be taken up into the mucosa. However, most of the proteins (contents) in the endosomes are degraded by lysozymes, and peptide fragments are expressed on the basolateral membrane (for example by major histocompatibility complex II) for recognition by surrounding lymphocytes. However, a very small percentage of intact protein can pass through the epithelial cells and be released at the basolateral membrane (Sanderson & Walker, 1993).

Dogma has underscored the idea that tight junctions are impermeable to macromolecules. It was thought that it is not possible (barring overt epithelial damage) for macromolecules to enter the mucosa via tight junctions. More recent studies provide evidence suggesting that given the appropriate signals tight junctions can open, allowing uptake of macromolecules. Cholinergic stimulation was shown to be important for the occurrence of this phenomenon (Phillips et al., 1987; Bijlsma et al., 1996).

1.1.4 Enteric Nervous System

The enteric nervous system is a third branch of the autonomic nervous system, although its organization resembles a “little brain,” and it can regulate the normal functioning of the gastrointestinal tract without outside innervation from the central nervous system.

Contained in the enteric nervous system are sensory nerves monitoring the luminal environment, a host of interneurons coordinating appropriate local reflexes and the secretomotor neurons which affect the physiology of the local cells (Wood, 1995). The enteric nervous system is estimated to have the same number of neurons as the spinal cord (Furness & Costa, 1987). For the intestines the enteric nervous system has two primary plexuses: the myenteric plexus (located between the longitudinal and circular muscle layers), responsible for regulating intestinal motility; and the submucosal plexus (located just inside the circular muscle layer), regulating mucosal activity, particularly epithelial ion transport and local blood flow (Furness & Bornstein, 1995).

The enteric nervous system contains an array of proven and putative neurotransmitters. A number of neurotransmitters have been observed, via immunostaining, to be colocalized in the same neurons. Acetylcholine is a primary neurotransmitter in the regulation of both the smooth muscle and the epithelium. Acetylcholine binds to muscarinic receptors on the basal membrane of crypt epithelium, signalling the calcium second messenger pathway, and ultimately causing chloride ion secretion at the apical membrane (Cooke & Reddix, 1994). Electrical field stimulation of nerves in intestinal preparations results in ion secretion by epithelium, mostly due to activation of cholinergic nerves, and indicated by an increase in short-circuit current (Perdue & Davison, 1986). Cholinergic receptor activation, by carbachol, was recently shown to increase epithelial permeability, permitting macromolecules, previously unable to cross, to pass through the tight junctions (Phillips et al., 1987; Bijlsma et al., 1996). Normal enteric nervous system activity maintains the basal passage of intact macromolecules across the epithelial barrier (Kimm et al., 1994). Other

important excitatory neurotransmitters located in the submucosal plexus include vasoactive intestinal peptide (which stimulates the production of cAMP, another second messenger that leads to active chloride ion secretion by epithelial cells) and substance P. Inhibitory neurotransmitters such as neuropeptide Y, somatostatin and opioids maintain the dynamic balance of epithelial functioning (Cooke & Reddix, 1994). For specific neuropeptide staining of the rat enteric nervous system in the small intestine and colon see Ekblad et al. (1987; 1988). See Table 1.1 below.

TABLE 1.1 Putative Enteric Neurotransmitters.

Neurotransmitter	Information	Species Where Identified
Acetylcholine (ACh)	neurons identified via ChAT activity; located in interneurons, several classes of enteric motor neurons and vagal input neurons; activating substance, prosecretory and increases GI smooth muscle activity	human, pig, cat, rat, mouse, guinea pig
adenosine triphosphate (ATP)	along with adenosine itself and other related nucleotides; modulates interneuron activity, in myenteric plexus probably submucosal as well; found in other lamina propria cells	guinea pig
calcitonin gene related peptide (CGRP)	colocalized with Sub P; some enteric motor neurons; extrinsic and intrinsic sensory afferent neurons	human, pig, rat, guinea pig
cholecystokinin (CCK)	colocalized with ACh, NPY, CGRP, SOM; excitatory interneural activity	human, dog, rat guinea pig
Galanin (GAL)	found in both plexuses and lamina propria; inhibitory action on excitatory neurons and possibly other cell types	human, pig, dog, rat, mouse guinea pig

Gastrin-releasing peptide (GRP)/ Bombesin (BOM)	include the neuromedin peptides B, C & U; affect intestinal fluid and electrolyte transport via other neurons and direct activity to cells; definitive role only known in stomach, localized throughout GI tract	dog, rabbit, pig, rat, guinea pig
γ -amino butyric acid (GABA)	stimulates inhibitory enteric neurons; decreases colonic motor activity in the colon	guinea pig
Histamine (HIS)	modulates neural activity; also localized in mast cells and enteroendocrine cells	guinea pig
5-hydroxy-tryptamine (5-HT)	also known as serotonin; directly affect cells, may modulate neural activity; found in myenteric neurons but also enteroendocrine cells and mast cells	human, pig
neuropeptide Y (NPY)	related to peptide YY and pancreatic polypeptide which are found mostly in endocrine cells, NPY exclusively neurons; colocalized in a number of different neurons adrenergic, cholinergic or VIPergic; has inhibitory activity on effector cells and other neurons	human, pig, dog, rabbit, rat, guinea pig
neurotensin (NT)	found in submucosal plexus neurons; stimulate secretion via activating sub-P or 5-HT; also found in enteroendocrine cells	sheep, dog
nitric oxide	located by NOS or NADPHase activity; localized in interneurons and inhibitory motor neurons; smooth muscle relaxant	dog, mouse, guinea pig
noradrenaline	only located in postganglionic sympathetic neurons; typical inhibitory activity, inhibiting neurotransmitter release of other enteric neurons and absorptive/antisecretory activity directly on epithelium	human, pig, dog, rat, guinea pig
opiates	include dynorphin (DYN), endorphin (END) and enkephalin (ENK); absorptive/antisecretory activity, also inhibit motility; also may inhibit release of prosecretory neurotransmitters like ACh from other enteric neurons; colocalized with VIP or postganglionic sympathetic nerves	human, pig, dog, rat, guinea pig

(continued on next page)

peptide histidine isoleucine (PHI)	also peptide histidine methionine, both share homology with VIP and come from the same precursor polypeptide; increases secretory activity like VIP but less potent	human, pig, cat, rat, guinea pig
pituitary adenylate cyclase activating peptide (PACAP)	stimulates other secretory neurons such as cholinergic and sub Pergic; related to VIP	human, pig, cat, rat, guinea pig
somatostatin (SOM)	a very potent antiseecretory agent along entire GI tract; acts directly on epithelium inhibiting secretion from a number of enteric agents not just neurotransmitters; also found in enteroendocrine cells; colocalized with prosecretory neurotransmitters, ACh, sub P, VIP	human, pig, dog, rabbit, rat, guinea pig
tachykinins	substance P (sub P), neurokinin A & B (NKA/NKB); substance most predominant; in sensory afferents and secretomotor neurons; colocalized with CGRP or ACh; stimulatory actions, ie. prosecretory or contract GI smooth muscle	human, pig dog, cat, rabbit, rat, mouse guinea pig
vasoactive intestinal polypeptide (VIP)	in both plexuses; localized in secretomotor neurons and colocalized with a number of different enteric neurotransmitters; enhance fluid and electrolyte transport, increased secretion and increased local blood flow	human, pig dog, rabbit, rat, guinea pig

modified from Cooke & Reddix (1994) and Furness & Bornstein (1995).

Although the enteric nervous system can act independently, the two classic branches of the autonomic nervous system, the sympathetic and parasympathetic branches, greatly influence the functioning of the enteric nervous system. In fact, about 90% of the ganglia in the submucosal plexus have direct innervation via the autonomic nervous system (Furness & Costa, 1987). The parasympathetic nervous system, which innervates most of the gastrointestinal tract via the vagus (except for the descending colon and rectum which are

innervated by the sacral nerves) increases the general activity of the gastrointestinal tract as well as the blood flow through the intestinal tissue. The sympathetic nervous system innervates the gastrointestinal tract by the thoraco/lumbar nerves with postganglionic nerves arising from the celiac and the superior and inferior mesenteric ganglia. Activation of the sympathetic nervous system generally slows down activity in the gastrointestinal tract and shunts blood away. The end neurotransmitter responsible for inhibiting epithelial cell secretion (and thus increasing electrolyte and water absorption) and relaxation of smooth muscles is noradrenaline (Dodd & Role, 1991). Local nerves in the submucosal plexus do not contain noradrenaline. Noradrenergic nerves innervate the submucosal plexus typically inhibiting neural activity (Cooke & Reddix, 1994). The two branches of the autonomic nervous system probably work in conjunction with one another, fine tuning the activity of the gastrointestinal tract, during normal everyday circumstances. See figure 1.5.

1.2 STRESS

1.2.1 Historical Perspective

The early work of Claude Bernard, in 1878, describing a “milieu intérieur,” followed by the work of Walter Cannon (1929 a,b) highlighting the concept of homeostasis (a dynamic equilibrium) and the purpose of the fight or flight response, provided the foundation and inspiration for Selye’s pioneering work. Selye’s (1936) first work described the classic triad of symptoms in response to stress: adrenal hypertrophy, thymic atrophy, and gastric ulceration. This concept has extended to our current understanding of stress responses: neuroendocrine regulation of the host’s reactions, impairment of the immune system, and

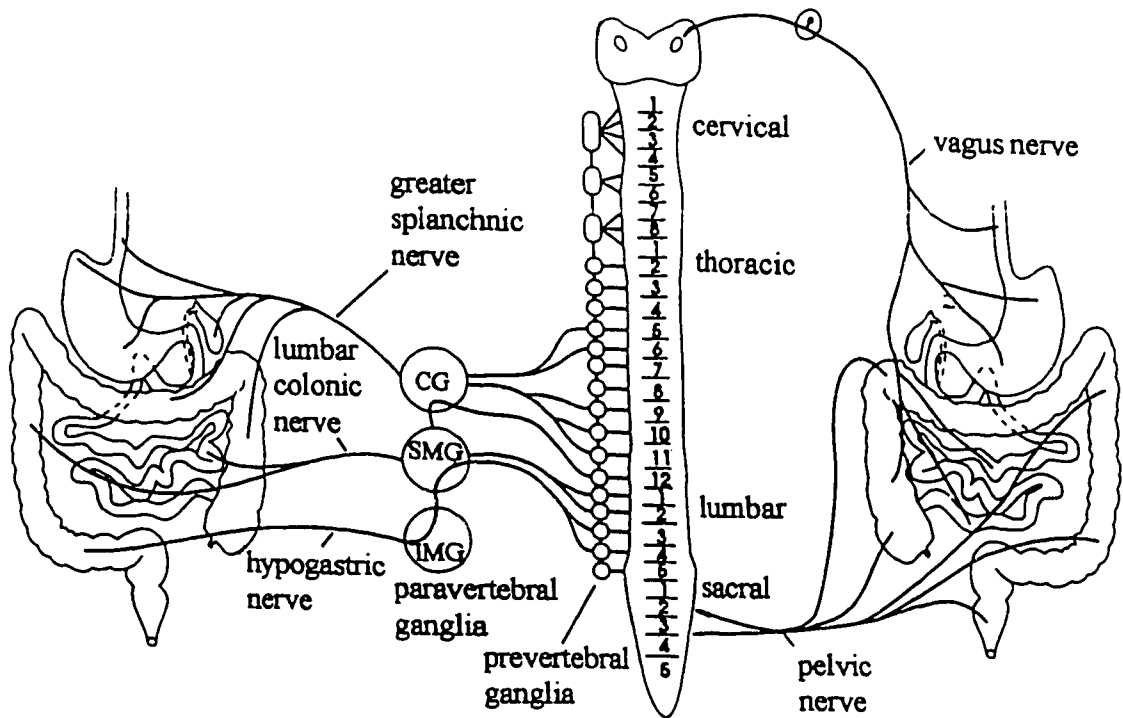


Figure 1.5 Autonomic innervation of the GI tract. Innervation of the gastrointestinal tract by the sympathetic nervous system is depicted on the left of the figure. The nerves originate from the thoracic and lumbar segments of the spinal column and branch through the prevertebral ganglia to the paravertebral ganglia. The splanchnic, lumbar colonic, and hypogastric nerves emerge from the celiac ganglion and the superior and inferior mesenteric ganglia to innervate the gastrointestinal tract. Innervation of the gastrointestinal tract by the parasympathetic nervous system is depicted on the right of the figure. The vagus nerve originates from the brain stem and the pelvic nerve from sacral segments of the spinal column. The vagus innervates most of the gastrointestinal tract while the pelvic nerve innervates the remaining portions (modified from Sengupta & Gebhart, 1994).

gastrointestinal disturbances. Selye (1946) subsequently expanded his original concept to the General Adaptation Theory. The General Adaptation Theory assumes that animals have a certain amount of energy that they can dedicate to dealing with environmental stressors and maintain a dynamic homeostasis (for more details regarding the General Adaptation Theory please see Appendix A).

1.2.2 Definition of Stress

“Stress is life and life is stress” (Selye, 1975). Selye defined stress, for scientific investigation, as the nonspecific response of the body to any demand (1950). This definition influenced the field of stress research as investigators studied how consistent the body’s responses were to a number of different stressors as well as examining the underlying mechanisms mediating the stress responses. However, observations contradicting Selye’s idea of stress began to emerge. Animals did not necessarily respond to different stressors in exactly the same fashion. With careful methodology (removing the emotional component of stress which accounts for Selye’s stress responses) it has been shown that different physical stressors cause different (sometimes exactly the opposite) physiological responses (Mason, 1975). For example, Mason (1968) found that circulating glucocorticoid levels increased when rhesus monkeys were subjected to a conditioned avoidance protocol. However, glucocorticoid levels decreased when the stress involved raising the environmental temperature 15°F above normal in 1°F increments every hour. Glucocorticoid levels did not change when the monkeys were food deprived (as long as the animals received fruit-flavoured nonnutritive cellulose pellets as a replacement). Measurement of catecholamines and

adrenocorticotropin hormone (ACTH, the hormone signalling the production and release of glucocorticoids from the adrenals) released in response to a number of different physical stressors showed a different pattern of release for these neuroendocrine hormones depending on which stressor was presented to rats (Kopin, 1995). An animal's prior history may also determine how it responds to stress (Mason, 1978). Rhesus monkeys, separated from their mothers at birth, were slowly habituated to a surrogate mother, either a dog or an inanimate object (a fur covered plastic toy). When the two groups of monkeys were stressed, they showed different patterns of responses compared to their basal values, including changes in behaviour and physiological responses (like body temperature) or levels of circulating "stress" hormones. Moberg (1985) determined that inclusion or exclusion of emotions impacted on the animal's interpretation of the stress. A fight or flight response (anxiety producing) involves a very strong adrenergic response, with stimulation of the cardiovascular system (for example high heart rate and high blood pressure). The resulting pathology from chronic stress in this circumstance is cardiovascular in nature: heart attack, stroke and/or vascular disease. On the other hand, if no active response is possible, a conservation-withdrawal strategy results (to preserve energy and detection) and the body responds with a strong glucocorticoid/parasympathetic response. If stress is persistent and the animal develops the condition of learned helplessness, the resulting pathologies are immunosuppression, depression, and gastrointestinal disorders. Finally, if an animal perceives control over its environment (being able to deal with its stressors) there is no pathology.

We are left with the accepted definition that stress is the body's response when homeostasis is threatened (Moberg, 1985; Stratakis & Chrousos, 1995). Most often, the

responses by the body are appropriate and necessary for survival. Researchers are interested in the stress responses when they are either inadequate or excessive and as a result lead to altered physiology that can cause pathology (Chrousos & Gold, 1995). Some influential researchers in the field of stress believe it impossible to come up with a single unifying definition of stress (i.e. symptoms or responses) that objectively indicates when an animal is in a state of poor 'wellbeing' compared to an animal that is able to cope with the stress (Overmier, 1988). Is it possible to assess an animal's wellbeing in response to stress? Examining only secreted neuroendocrine mediators is not sufficient. A number of parameters can be examined to develop a pattern of stress responses. The impact of the neuroendocrine systems during stress responses on such functions as reproduction, growth, metabolism, or the impact on the cardiovascular, gastrointestinal or immune systems, gives a better idea of an animal's state of wellbeing (Stratakis & Chrousos, 1995). A key goal is to understand how stress contributes to disease; how stress creates a prepathological state (for more information regarding a stress-induced prepathological state please see Appendix A).

1.2.3 Central Mediation of Stress

The central nervous system initiates the stress response. Midbrain structures coordinate and integrate environmental information from the senses along with psychological information from higher centres of the brain including cognitive material from the cortices, via the hippocampus, and emotional details from the mesocorticolimbic system. These midbrain structures are then able to mediate stress responses throughout the brain and the body via the neuroendocrine systems (Chrousos & Gold, 1995). Complicated sensory

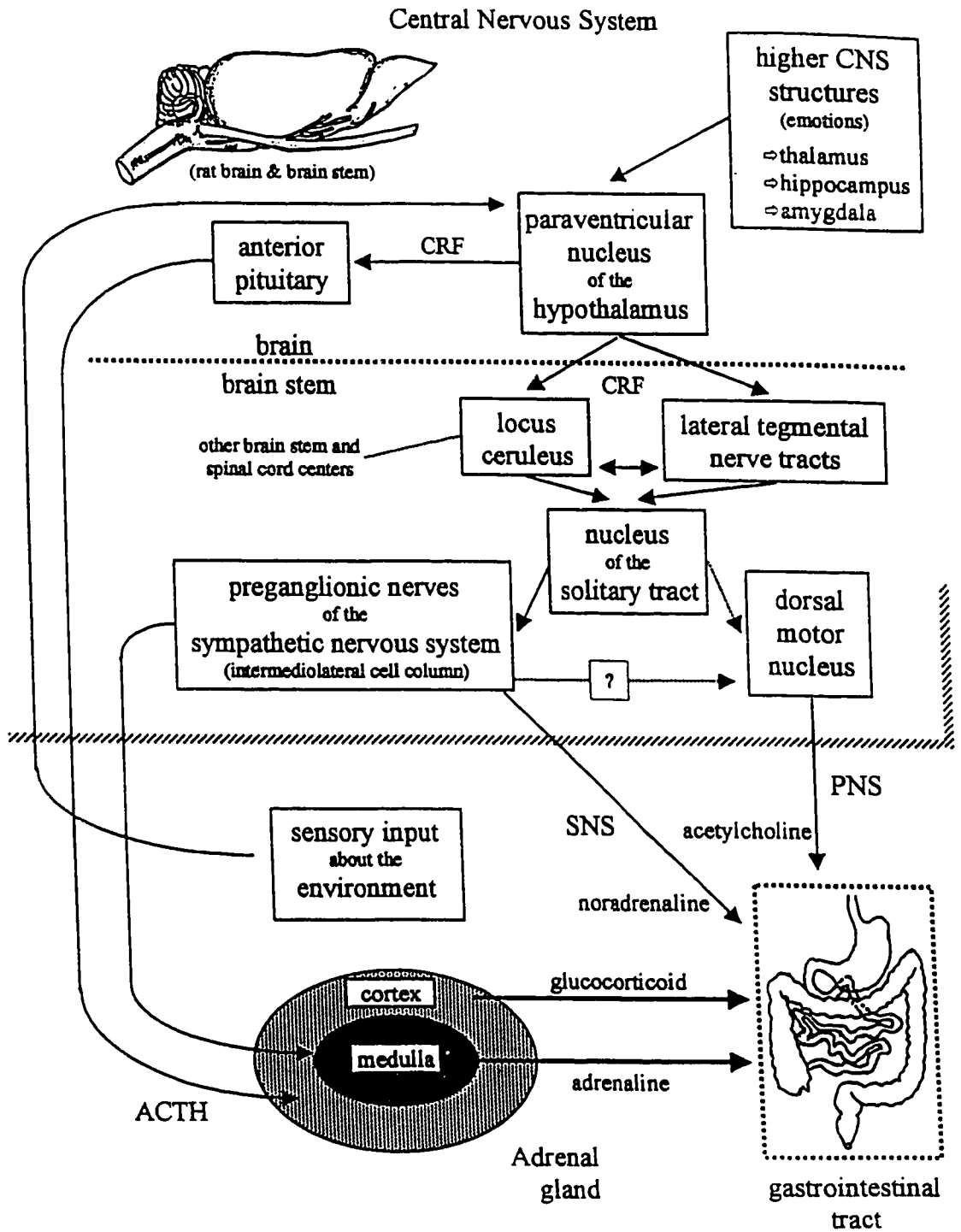
information about abrupt changes in the environment is relayed and processed at many levels of the central nervous system. The thalamus provides coordination of the many neural inputs, and along with the limbic system, particularly the amygdala, which is critical for emotional processing in the brain, signals the hypothalamus to initiate an appropriate response to an environmental stressor. The paraventricular nucleus of the hypothalamus produces and releases corticotropin releasing factor in response to stress. Corticotropin releasing factor is released into the blood and activates the anterior pituitary to release adrenocorticotropin hormone into the blood. This in turn signals the cortex of the adrenal gland to release glucocorticoid hormones into the blood. Circulating glucocorticoids have profound effects on a number of organs and physiological processes throughout the body, and increased levels of plasma glucocorticoids have been typically used in the past to determine if animals are in a state of stress. Corticotropin releasing factor projections also extend to the locus ceruleus and the lateral tegmental nucleus. These two nuclei contain the only noradrenergic neurons in the brain stem. These nuclei have connections throughout the brain stem including the sympathetic preganglionic neurons in the intermediolateral cell column and the nucleus of the solitary tract. Neural connections are also present to the dorsal motor complex, the nucleus for preganglionic neurons of the vagus. Corticotropin releasing factor is a key central mediator of the stress response, activating the hypophyseal adrenal axis to release glucocorticoids and activating the sympathetic nervous system. Corticotropin releasing factor may also activate the parasympathetic nervous system (Dunn & Berridge, 1990; Johnson et al., 1992; Role & Kelly, 1991). The parasympathetic rebound theory indicates that parasympathetic involvement, following sympathetic activation, is critical for a number of

observed gastrointestinal responses to stress, especially stress-induced gastric ulceration (Manneker, 1976; Ray et al., 1987). See figure 1.6.

1.2.4 Stress & The Gastrointestinal Tract

Since Selye's (1936) first work with stress, a number of studies have examined stress-induced gastric ulceration; for a review see Glavin (1980). More specifically, studies have examined central mediators of stress and how they are involved in causing the ulceration (Bhargava et al., 1980); involvement of the autonomic nervous system and the neuroendocrine system (Ray et al., 1987); the local responses in the stomach such as gastric acid production, decreased gastric blood flow, impaired gastric motility and delayed gastric emptying, as well as the local mediators in the stomach responsible for these physiological responses (Basso et al., 1983; Kitajima et al., 1991; Takeda et al., 1992; Yano et al., 1978). Other studies examined endogenous and exogenous mediators that help control the ulceration and/or initiate repair mechanisms following damage (Hanson & Brodie, 1960; Nosalova et al., 1991). Other studies have analysed the physical and psychological properties of the stressor that contribute to ulcerogenesis (Bohmelt et al., 1994; Senay & Levine, 1967). Factors that make an individual more or less susceptible to stress-induced ulceration include gender, age, any form of previous history including prenatal stress, genetic background, etc. (Pare, 1989 a,b; Shichijo et al., 1991; Sines, 1959). Clearly, an immense wealth of information may be gleaned from such a body of work, and will aid in understanding stress responses in other parts of the gastrointestinal tract.

Figure 1.6 Central mediation of GI stress responses. The paraventricular nucleus of the hypothalamus is signalled when the body is facing stressful circumstances. This complex information comes from higher brain structures involved in determining conscious thoughts and emotions. A major relay and filter for this information is the thalamus. Other structures of the limbic system, like the hippocampus and amygdala, are also involved. Sensory information about the environment also contributes to triggering a stress response. The hypothalamus signals the anterior pituitary, via corticotropin releasing factor (CRF) released into the blood, to deliver adrenocorticotropin hormone (ACTH) into the blood. ACTH signals the adrenal cortex to synthesize and release glucocorticoids, which will circulate in the blood and will affect the gastrointestinal tract. The hypothalamus will also stimulate the locus ceruleus and lateral tegmental nerve tracts via CRFergic nerves. These areas will activate the sympathetic nervous system (SNS) via the nucleus of the solitary tract. The SNS will directly affect the gastrointestinal tract, but it also signals the adrenal medulla to release adrenaline into the blood which will emphasize the SNS activity. The parasympathetic nervous system (PNS) is involved in gastrointestinal responses to stress; however, the exact mechanism for stimulating PNS activity is not clear. There are neural connections between PNS centres, SNS centres and the nucleus of the solitary tract.



Stress also affects the motility apparatus of the gastrointestinal tract. Stress typically suppresses gastric activity and emptying as well as motility of the proximal small intestine, while stress will increase colonic motility, sometimes resulting in diarrhea. A number of studies have examined the central, peripheral, and local mediators responsible for the perturbations in normal smooth muscle activity in the gastrointestinal tract (Almy et al., 1949; Bueno et al., 1989; McRae et al., 1982; Monnikes et al., 1992; Williams et al., 1988; Wittman et al., 1990).

In comparison to the research exploring the effects of stress on gastric ulcers and intestinal motility, very few studies have examined stress effects involving the intestinal mucosa, particularly the intestinal epithelium. From those reports that have addressed the topic of stress affecting the intestinal epithelium, most studies examined stress-induced increases in the secretory activity of intestinal epithelial cells, particularly salt and water secretion (Barclay & Turnberg, 1987; Barclay & Turnberg, 1988 a,b; Empey & Fedorak, 1989; Lenz, 1989), but also colonic mucin secretion from goblet cells (Castagliuolo et al., 1996). Other research results have shown that severe stress caused increased intestinal epithelial permeability in the small bowel (Carter et al., 1990; Rhodes & Karnovsky, 1971) or colonic mucosal damage (Andrianopoulos et al., 1986), as well as increased production of duodenal ornithine decarboxylase (Wang & Johnson, 1989), an enzyme critical in the production of polyamines and necessary for repair of damaged tissue. A tremendous amount of research is still required in order to understand how stress affects the intestinal mucosa. The need for this information, and its potential importance to enteric disease, was the impetus for the current investigations.

1.3 STUDY PLAN

The purpose of my project was to develop an animal model to examine the effects of acute stress on intestinal epithelium and to gain insights into the mechanisms involved. I documented alterations in epithelial physiology in response to restraint stress in Wistar Kyoto rats, known to be a very stress susceptible strain. The stress-induced responses of the intestinal epithelium included increased chloride ion secretion and increased permeability to ions and small molecules. I tested the hypothesis that cholinergic nerves were involved in mediating the stress effects by pretreating rats with atropine to block cholinergic stimulation at muscarinic receptors. I expanded the model to document that tissues from stressed rats were also more permeable to macromolecules. Finally, I examined the effects of stress on epithelium from different parts of the gastrointestinal tract. I also determined that cholinergic nerves and corticotropin releasing factor were mediating colonic stress responses. My studies lend support to the concept that stress can exacerbate intestinal disease by enhancing uptake of luminal macromolecules, such as antigens and microbial products.

The following work, my first publication, addresses the effects of acute stress on intestinal (jejunal) epithelial physiology. The paper outlines my experimental model I examined, as well as the physiological parameters of the intestinal epithelium.

Chapter 2

**Acute stressors stimulate ion secretion and increase
epithelial permeability in rat intestine.**

P.R. Saunders, U. Kosecka, D.M. McKay and M.H. Perdue.

American Journal of Physiology, 267: G794-G799, 1994.

Copyright held by American Physiological Society

NOTE TO USERS

**Page(s) missing in number only; text follows.
Microfilmed as received.**

34

This reproduction is the best copy available.

UMI

ABSTRACT

Wistar-Kyoto rats were subjected to 4 h restraint stress (RS) or cold restraint stress (CRS), and jejunal tissues were examined in Ussing chambers for alterations in transport functions compared with tissues from unstressed control rats. The baseline short-circuit current (I_{sc}) was significantly elevated in tissues from RS (~50%) and CRS (100%). Substitution of Cl⁻ eliminated the abnormality, suggesting that stress stimulates Cl⁻ secretion. Electrical transmural stimulation of enteric nerves caused a transient increase in I_{sc} in all tissues. The magnitude of this response was significantly less in tissues from CRS than from control rats; however, the ability of the epithelium to secrete in response to exogenous stimulation with bethanechol or vasoactive intestinal polypeptide was unimpaired, implicating a neural change. Tissue conductance was higher in jejunum from RS and CRS rats than from controls. Increased intestinal epithelial permeability in stressed rats was confirmed by significantly greater fluxes of the inert radiolabelled probes, [³H]mannitol and ⁵¹Cr labelled EDTA. No structural changes were observed. We conclude that acute stressors have profound effects on intestinal epithelial physiology, stimulating ion secretion and reducing barrier function.

2.1 INTRODUCTION

A variety of clinical evidence indicates that anxiety and stress are associated with

gastrointestinal dysfunction, including abdominal pain and diarrhea [2,3,12,16,18]. In one of the earliest recorded studies in this area, Beaumont [6] reported that normal gut function was disturbed in a patient with a gastric fistula during periods of emotional stress. Selye introduced stress research in animal models [29] and characterized induction of gastric ulcer as a classical response to stress [30]. Experimental studies have demonstrated stress-related altered colonic motility in both healthy subjects and patients with irritable bowel syndrome [1,2,19]. In addition, patients with inflammatory bowel disease indicate that relapses are frequently preceded by periods of stress [10].

Only a few studies have examined the effect of stress on mucosal function in the small or large intestine. Alterations in fluid and electrolyte absorption in humans [3-5] and rats [11] have been reported. Evidence has also been presented to indicate that stress increases ornithine decarboxylase activity and decreases overall DNA synthesis in intestinal mucosa [33]. In this study, we examined parameters of epithelial ion transport and permeability in intestinal mucosa from rats stressed by restraint with or without cold and compared results with those in controls. We found that stress has profound effects on both functions, stimulating ion secretion and impairing the barrier function of the gut.

2.2 METHODS

2.2.1 Animal Model

Wistar-Kyoto rats (male, mean wt 150 g), a stress-susceptible strain [20,21], were purchased from Charles River Breeding Laboratories (St. Constant, Quebec, Canada). The rats were maintained in a normal light/dark cycle, received food and water ad libitum, and

were handled daily for 2 wk by the same researcher. Rats were fasted overnight for 14 h before being subjected to the stress protocol.

For restraint stress (RS), the rat was placed in an adjustable restraining device (6 x 21.5 cm) for 4 h at 20°C. For cold restraint stress (CRS), the device was placed in an 8°C room. After the defined period, the rat was removed from the device and anaesthetized with urethane (Aldrich Chemical, Milwaukee, WI). The rat was weighed, and its temperature was monitored by a rectal probe. If necessary, the rat was warmed via a thermally controlled heating pad until its core body temperature reached 36°C before the experiment was begun. Then a blood sample was obtained for later measurement of corticosterone by radioimmune competition assay (Diagnostic Products, Los Angeles, CA). The abdominal cavity was opened, and a 15 cm segment of jejunum, beginning 5 cm distal to the ligament of Treitz, was removed for Ussing chamber studies and histological evaluation.

Control rats were handled in the same manner as experimental rats but were maintained in their home cages at all times before study. To determine if fasting is a minor stressor, we compared results in both fasted and nonfasted control rats. Experiments were approved by the Animal Care Committee at McMaster University.

2.2.2 Ussing Chamber Studies

The excised segment of jejunum was stripped of longitudinal muscle and myenteric plexus, leaving the submucosal plexus intact [22]. The tissue was then opened along its mesenteric border and placed in 37°C oxygenated Krebs buffer, pH 7.35 ± 0.02 . Four adjacent pieces of stripped jejunum from each rat were mounted in Ussing-type chambers that

contained agar-salt bridges to monitor potential difference (PD) and inject short-circuit current (I_{sc}). The chambers included stimulating electrodes on opposite sides of the tissue [23]. The chamber opening exposed 0.6 cm² of serosal surface area to 10 ml of oxygenated Krebs buffer that contained (in mM) 115 NaCl, 1.25 CaCl₂, 1.2 MgCl₂, 2.0 KH₂PO₄, and 25 NaHCO₃, pH 7.35 ± 0.02, at 37°C. In addition, the serosal buffer contained 10 mM glucose, and the mucosal buffer contained 10 mM mannitol (final solution 310 mosM). The tissue was clamped at 0 V with the use of a W-P Instruments automatic voltage clamp (Narco Scientific, Downsview, Canada). The I_{sc} (in μ A) was recorded continuously, and PD measurements (in mV) were taken at intervals during the experiments. Conductance (G, in mS/cm²) was calculated using values of PD and I_{sc} .

Baseline I_{sc} was determined as an indication of the ion transport state of the tissue. Some experiments were conducted using Cl⁻-free buffer (equimolar substitution of isethionate and acetate ions for Cl⁻) or bicarbonate-free buffer (equimolar substitution of piperazine ethanesulfate ions for bicarbonate ions plus 1 mM acetazolamide to inhibit carbonic anhydrase [7]). Electrical transmural stimulation (TS) of enteric nerves was conducted by passing rectangular current pulses from one side of the tissue to the other via stimulating electrodes and a Grass stimulator (Grass Medical Instruments, Quincy, MA) [24]. The stimulus parameters were 10 mA, 10 Hz, and 0.5 ms pulse width delivered in trains for a total of 5 s. We have previously shown that the tissue response to TS is eliminated by neural blockade with tetrodotoxin [23]. In specific experiments, the muscarinic agonist, bethanechol, or vasoactive intestinal peptide (VIP; Sigma Chemical, St. Louis, MO) was added to the serosal buffer in cumulative concentrations from 10⁻⁶ to 10⁻³ M or 10⁻⁹ to 10⁻⁶ M, respectively, after

pretreatment with 10^{-6} M tetrodotoxin (Sigma). Responses (ΔI_{sc}) were measured as the difference between basal I_{sc} and the maximal I_{sc} after TS or secretagogue addition.

Flux studies for Na^+ and Cl^- were conducted in tissue pairs in which 5 μCi of $^{22}\text{Na}^+$ and 2.5 μCi of $^{36}\text{Cl}^-$ (DuPont, Wilmington, DE) were added to the mucosal (luminal) side or the serosal side for each pair. Four 1.0-ml samples were taken from the "cold" side at 15-min intervals beginning after a 20-min equilibration period, while two 50- μl samples (to calculate specific activity) were taken from the "hot" side at the same time as the first and last samples were taken from the "cold" side. Radioactivity was measured in beta and gamma counters, and the fluxes were calculated by standard formulas. Fluxes of ions crossing the mucosa were expressed as $\mu\text{mol}/\text{cm}^2/\text{h}$, and net movement was calculated as the difference between the unidirectional fluxes [25].

The permeability of the jejunal mucosa to two inert probes, [^3H]mannitol and ^{51}Cr -labelled EDTA (^{51}Cr -EDTA), was measured by adding them to the mucosal buffer and taking samples from "hot" and "cold" sides as indicated above. [^3H]mannitol (Sigma Chemical) was added at 2.4 $\mu\text{Ci}/\text{ml}$; ^{51}Cr -EDTA (Radiopharmacy, McMaster-Chedoke Hospital, Hamilton, Canada) was added at 6 $\mu\text{Ci}/\text{ml}$. Equivalent concentrations (10 mM for mannitol and 0.85 μM for ^{51}Cr -EDTA) of nonradioactive compounds were present in the serosal buffer. The radioactivity of [^3H]mannitol was measured in a beta counter; ^{51}Cr -EDTA was measured in a gamma counter. Fluxes of probes crossing the mucosa were expressed as $\text{nmol}/\text{cm}^2/\text{h}$.

2.2.3 Histology

Segments adjacent to those taken for chamber studies were removed, opened along

the mesenteric border, flattened, and stapled on a card that was placed in 10% neutral buffered Formalin. Fixed tissue was sectioned and stained with haematoxylin and eosin. Villus height, villus width (one third from the top of the villus), crypt depth, and epithelial integrity were measured using a calibrated eyepiece on 7-10 well-oriented villus-crypt units per coded (rat group unidentified) section. The following scale was used to assign a damage score per section allowing the epithelial integrity to be rated: 0 intact epithelium with no cell sloughing; 1, some cells or cellular debris in lumen and/or minor edema (separation of epithelium from villus core); 2, minor breaks at villus tip and/or obvious epithelial cell damage; 3, moderate number of cells in lumen and major breaks in the epithelium; and 4, denuded villi. Average values per section were used to calculate group means.

2.2.4 Statistics

Statistical analyses were performed using analysis of variance with Newman-Keuls as a subsequent multiple comparison test and Student's t-test or Mann-Whitney for individual comparisons. Statistical significance was defined at $p < 0.05$.

2.3 RESULTS

2.3.1 Corticosterone

Plasma corticosterone levels were significantly increased ($p < 0.001$) in CRS rats (755.6 ± 16.9 ng/ml) and RS rats (727.3 ± 18.0 ng/ml), compared with unstressed fasted rats (523.1 ± 15.9 ng/ml) and fed control rats (399.2 ± 11.5 ng/ml), $n = 14-29$ in each group. Also, plasma corticosterone levels were significantly increased ($p < 0.001$) in fasted rats

compared with fed control rats.

2.3.2 Ion Transport

The baseline I_{sc} (indication of ion transport state) was increased in intestine of stressed rats (Figure 2.1A). At 15 min, values for CRS and RS rats were significantly different ($p < 0.001$) from values in control and fasted rats. Cold significantly enhanced the transport abnormalities. I_{sc} values in control rat intestine decreased slightly over time, whereas values in stressed rat intestine remained elevated.

To examine the role of Cl^- secretion in the increased baseline I_{sc} tissues from stressed and control rats were bathed in either Krebs or Cl^- -free buffer. Replacement of Cl^- did not affect the baseline I_{sc} of tissues from control rats (Figure 2.1B). However, the increased I_{sc} of tissues from CRS and RS rats was significantly reduced ($p < 0.05$) in Cl^- -free buffer. In the absence of Cl^- , the baseline I_{sc} of tissues from CRS and RS rats was not significantly different from the baseline I_{sc} of tissues from control rats. Table 2.1 shows that unidirectional fluxes of both Na^+ and Cl^- were significantly increased in tissue from stressed rats compared with controls and net Cl^- movement was reversed from absorption to secretion. Bicarbonate-free buffer made no difference in the baseline I_{sc} in tissues from stressed or control rats (data not shown).

Electrical stimulation of enteric nerves in the tissues resulted in a rapid increase in I_{sc} that returned to the baseline value in less than 5 min. Tissues from CRS rats had a significantly reduced ($p = 0.001$) response to TS compared with tissues from fed or fasted control rats (Figure 2.2A). However, there was no significant difference in the I_{sc} response

Figure 2.1 A: Baseline short-circuit current (I_{sc}) of jejunal tissues from rats: control (+), fasted (\diamond), restraint stress (RS; \circ), and cold restraint stress (CRS; \square). The no. of rats in each group was 11-15. At 10 min, mean I_{sc} in intestine from CRS rats was significantly different ($p < 0.05$) from I_{sc} in control and fasted groups; from 15 min, CRS and RS values were significantly different ($p < 0.05$) from other groups. Time 0, time at which all tissues were mounted in Ussing chambers 45 min after rats were anaesthetized. B: baseline I_{sc} 15 min after mounting tissues from control, RS and CRS rats in Krebs buffer (open bars), or chloride-free buffer (solid bars). The no. of rats in each group was 5. * is $p < 0.05$ for tissues in chloride-free buffer compared with tissues in Krebs buffer. Baseline I_{sc} of tissues from CRS and RS rats in normal buffer was significantly increased ($p < 0.05$) compared with tissues from controls. Values are means \pm SEM for A and B.

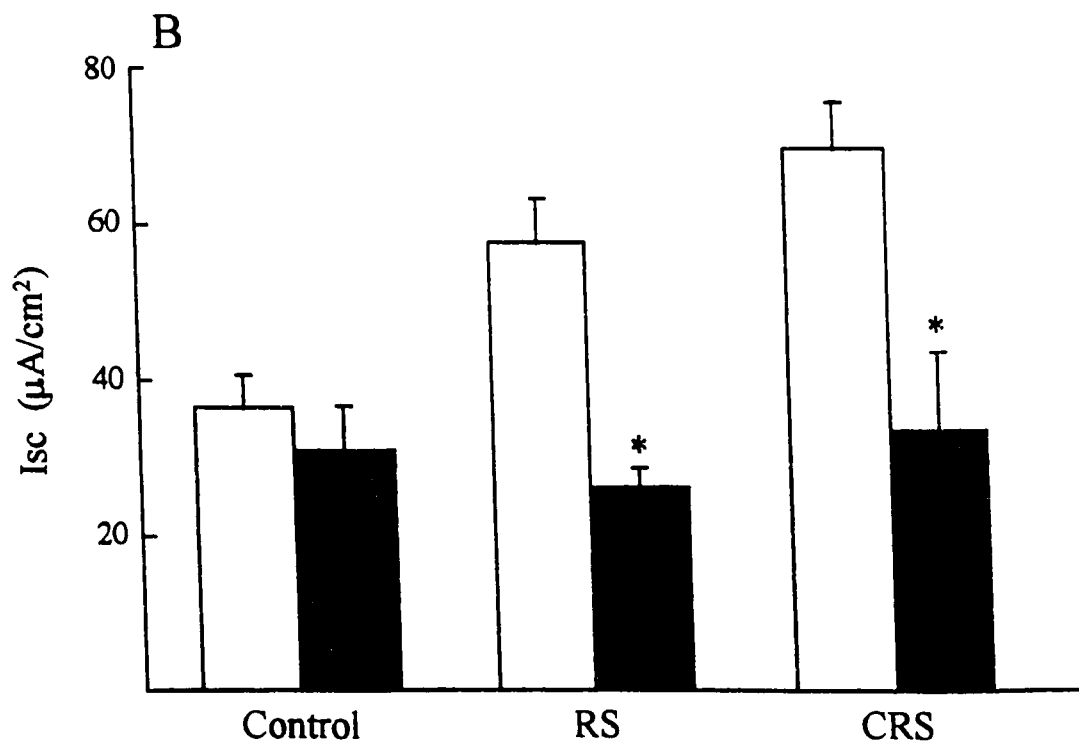
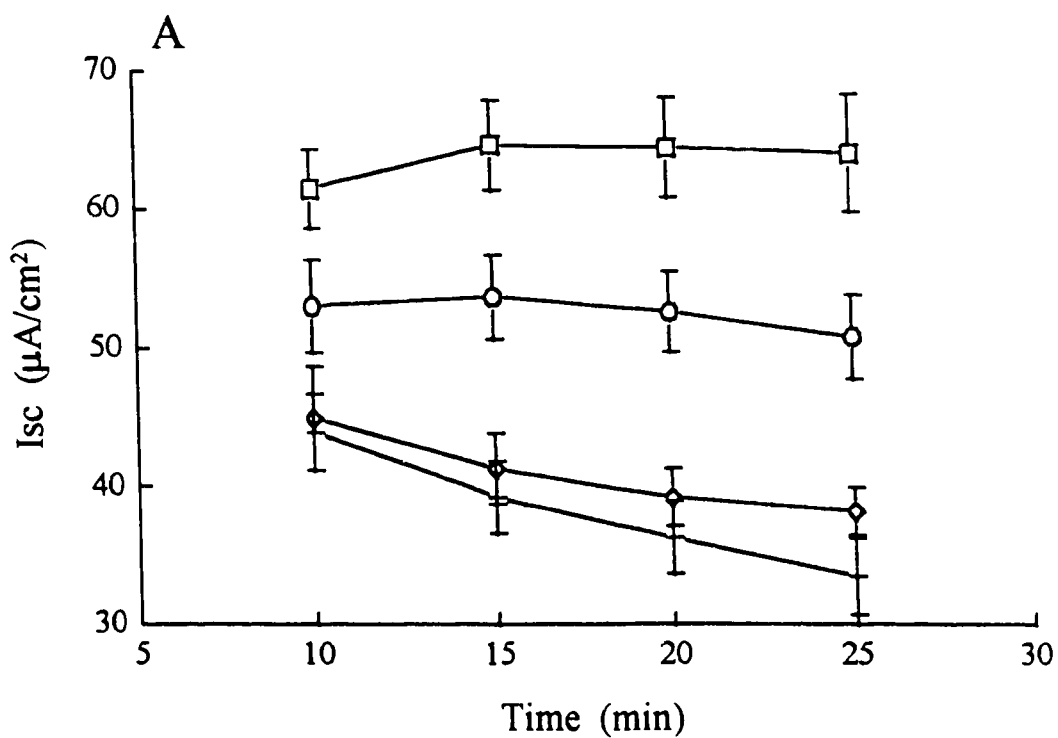
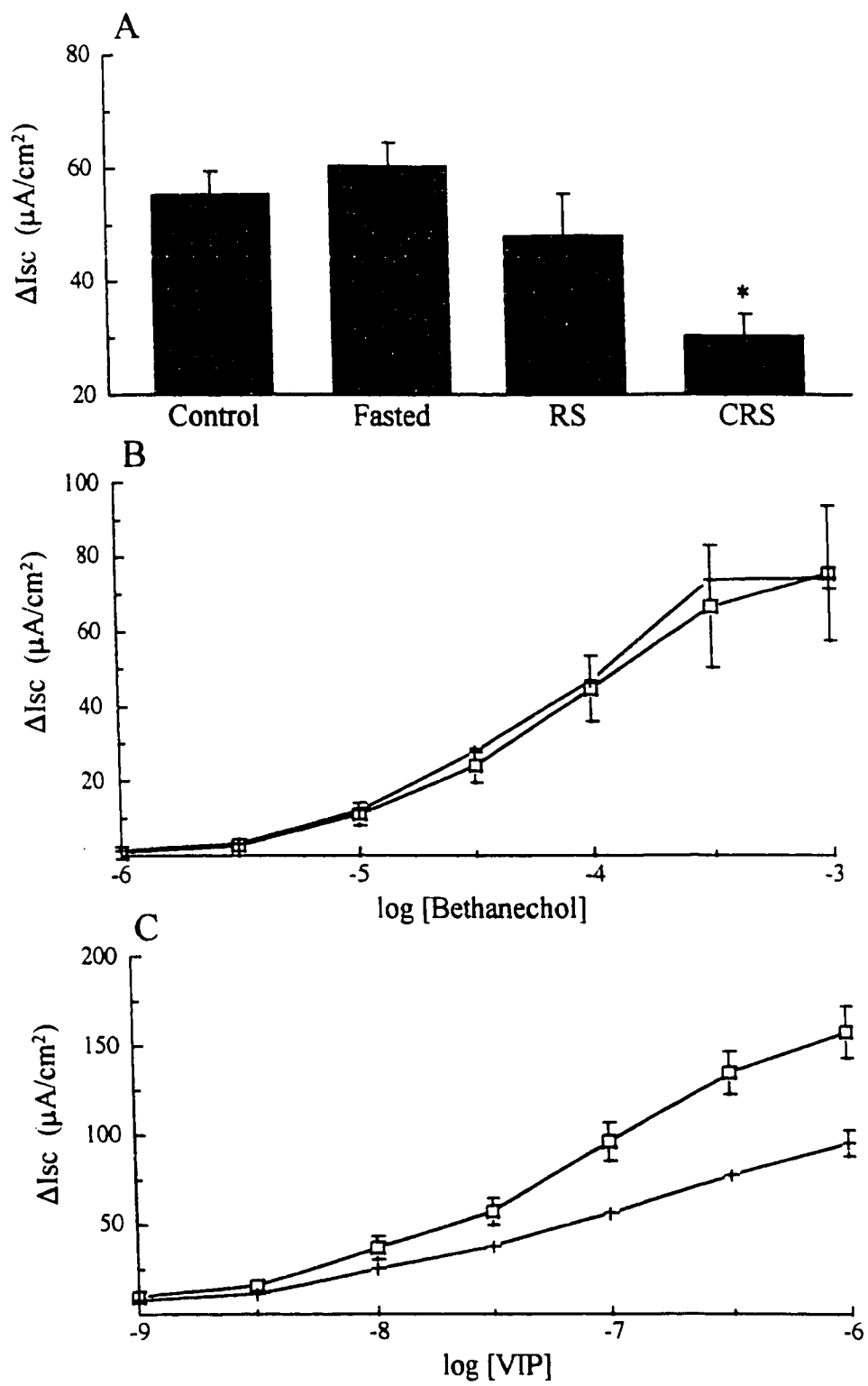


Figure 2.2 A: Increase in I_{sc} in response to transmural stimulation (TS) in tissues from controls, fasted, RS, and CRS rats. The no. of rats in each group was 10-16. * is $p < 0.05$ compared with controls. B: Increase in I_{sc} in response to addition of bethanechol in tissues from control rats (+) or CRS rats (\square). Tissues from at least 4 rats were used for each point. C: Increase in I_{sc} in response to addition of VIP in tissues from control rats (+) or CRS rats (\square). Tissues from at least 4 rats were used for each point. Values from -7.5 to $-6 \log [VIP]$ were significantly increased ($p < 0.05$) in tissues from CRS compared with controls. Values are means \pm SEM for A-C.



to bethanechol for tissues from CRS rats compared with controls (Figure 2.2B). The I_{sc} response to VIP was also not impaired in tissues from CRS rats (Figure 2.2C) but was actually increased at concentrations of 3×10^{-8} to 10^{-6} M.

TABLE 2.1 Na^+ and Cl^- fluxes in jejunal tissues from control and CRS rats

	Na^+ , $\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$			Cl^- , $\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$		
	J_{m-s}	J_{s-m}	J_{net}	J_{m-s}	J_{s-m}	J_{net}
Control	10.3±0.6	9.0±0.2	1.3±0.4	12.9±0.7	11.2±0.3	1.7±0.8
CRS	16.3±0.8*	15.2±0.5*	1.6±0.5	16.3±0.9*	18.8±0.8*	-2.1±0.4*

Values are means \pm SEM; n = 6 tissue pairs in each group. J_{m-s} , mucosal to serosal flux; J_{s-m} , serosal to mucosal flux; J_{net} , total flux; CRS, cold-restraint stress. * is $p < 0.02$ compared with controls.

2.3.3 Permeability

The conductance values for tissues from all groups of rats were very consistent over time. However, tissues from both CRS and RS rats had significantly increased ($p < 0.001$) conductance values compared with tissues from fed or fasted control rats (Figure 2.3A). Fasting alone increased tissue conductance compared with values in fed control rats. In addition, conductance abnormalities were significantly enhanced by cold in stressed rats.

Two radiolabelled inert probes, [^3H]mannitol and ^{51}Cr -EDTA, were used to assess epithelial permeability. There was a significant increase ($p = 0.008$) in mannitol flux for tissues from both CRS and RS rats compared with controls (Figure 2.3B), and a significant increase ($p = 0.018$) in ^{51}Cr -EDTA flux for tissues from CRS rats compared with tissues from controls (Figure 2.3C).

2.3.4 Histology

Morphometric analysis of tissue architecture revealed no significant differences between tissues from stressed rats versus control rats. In addition, examination of epithelial integrity revealed no differences (Table 2.2). Both groups had an identical mean damage score of 0.5, indicating no identifiable abnormalities.

TABLE 2.2 Morphometric measurements of jejunal tissues

	Villus Length μm	Crypt Depth μm	Villus Width μm	Damage Score
Control	485 ± 9	203 ± 2	96 ± 3	0.5 ± 0.3
CRS	475 ± 22	208 ± 5	97 ± 5	0.5 ± 0.3

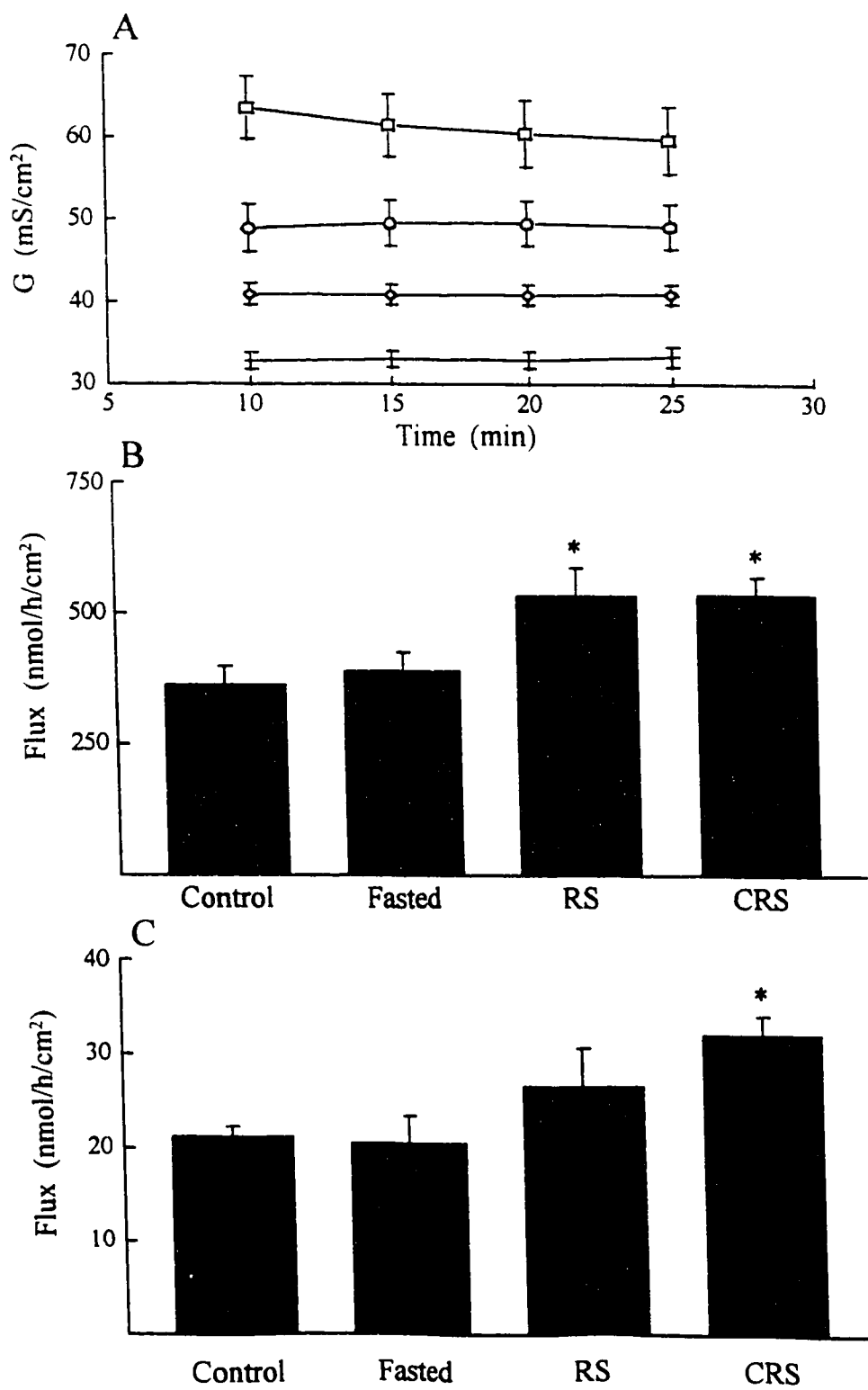
Values are means \pm SEM; 7-10 villus crypt units were measured for each tissue section; n = 4 rats in each group.

2.4 DISCUSSION

This study has identified that acute stressors cause profound changes in epithelial physiology of the small intestine. These changes include stimulation of ion secretion, increased permeability, and altered neural function.

Intestinal preparations from the two groups of stressed rats (CRS and RS) had a significantly increased baseline I_{sc} compared with those from fed controls and fasted rats. The increased I_{sc} in CRS and RS intestine was due to increased Cl^- secretion, since the elevated baseline I_{sc} returned to normal when chloride ions were replaced by other anions in the buffer. In addition, radioactive flux studies provided direct evidence for net Cl^- secretion. These studies are compatible with *in vivo* studies in rats in which CRS caused fluid accumulation

Figure 2.3 A: Baseline conductance (G) of jejunal tissues from rats: fed controls (+), fasted (\diamond), RS (\circ), and CRS (\square). The no. of rats in each group was 12-15. At the 10- and 15-min time points, all groups were significantly different ($p < 0.05$) from each other; at the 20- and 25-min time points, the RS and CRS groups were significantly different ($p < 0.05$) from the other groups. Time 0, time at which all tissues were mounted in Ussing chambers 45 min after rats were anaesthetized. B, C: Flux values across jejunal tissues from control and stressed rats for mannitol (B) and ^{51}Cr labelled EDTA (C). The no. of rats in each group was 4-6. * is $p < 0.05$ compared with control values. Values are means \pm SEM for A-C.



in the ileum and colon [11]. *In vivo*, increased intestinal Cl^- secretion is a driving force for the movement of positively charged ions, thus increasing the osmotic pressure and attracting water into the lumen. In humans, psychological stress caused the reversal of Na^+ and Cl^- absorption to secretion [3], and cold pain and exercise stress significantly decreased electrolyte and water absorption [4,5].

In intestine from all rats, neural stimulation caused transient increases in I_{sc} as we have earlier reported [24]. The magnitude of the I_{sc} increase to TS was reduced in intestinal tissues from stressed rats and significantly decreased in CRS rats compared with controls. TS releases neurotransmitters from the submucosal plexus neurons that act directly or indirectly on enterocyte receptors to stimulate anion secretion and thus increase I_{sc} [15,23]. In several species, a large component of the TS response is due to release of acetylcholine, which acts on epithelial muscarinic receptors [15,23,24]. To determine if the epithelial capacity to respond to such a stimulus was impaired, we added bethanechol, a cholinergic muscarinic agonist that stimulates Cl^- secretion via increasing intracellular Ca^{2+} [9], during neural blockade with tetrodotoxin. The change in I_{sc} in response to the addition of bethanechol was unaltered by stress. The change in I_{sc} in response to the addition of another putative neurotransmitter, VIP, which stimulates Cl^- secretion via increasing intracellular adenosine 3',5'-cyclic monophosphate [9], was not impaired, but was actually significantly greater. The reason for the increased response is not clear at this time and will require further investigation. However, our findings suggest that the reduced I_{sc} response to TS in stressed rats was not due to an epithelial defect at either the receptor or intracellular signalling level. The defect may have been due, at least in part, to changes in mucosal nerves, possibly caused by stress-

induced depletion of stimulatory neurotransmitters such as acetylcholine. Other possibilities include increased release of inhibitory neurotransmitter(s) or nerve damage as a consequence of the stress procedure or cold. However, the fact that rats were warmed to physiological temperature before intestinal function was studied makes this latter possibility less likely. Studies currently in progress support our hypothesis that functional epithelial abnormalities are mediated via neural mechanisms.

The increased conductance of intestine from stressed rats indicates dramatic and consistent effects on epithelial permeability to ions. In addition, the probe uptake studies demonstrated that the permeability of the tissue to larger molecules was also affected by exposure of rats to acute stressors. The jejunal epithelium from stressed rats was leakier to molecules at least the size of mannitol (mol wt 183 Da; diam 6.7 Å) and ⁵¹Cr-EDTA (mol wt 343 Da; diam 11.5 Å). Mannitol is reported to diffuse across the epithelium via intracellular and paracellular pathways, whereas ⁵¹Cr-EDTA crosses the epithelium by the paracellular route [14,32]. Previous work from this laboratory showed that ⁵¹Cr-EDTA is a valid marker of intestinal permeability since its uptake reflects that of macromolecular protein antigens [27]. Increased permeability of the jejunum during periods of stress would result in increased uptake of luminal antigens and enterotoxins and therefore could theoretically initiate or exacerbate inflammatory conditions of the gut. Indeed, patients with inflammatory bowel disease confirm that periods of stress often precede relapses [10].

The mechanism by which stress decreases barrier function has not been determined but might also relate to neural influences, since cholinergic stimulation has previously been shown to increase paracellular passage of horseradish peroxidase [26]. A theory in gastric

ulcer research proposes that the majority of the effects of stress are due to rebound stimulation of the parasympathetic nervous system following sympathetic activation. Whether this is a consequence of “sympathetic exhaustion” [13] or an interaction between the sympathetic and parasympathetic nervous systems [28] is still controversial.

Examination of histological sections by light microscopy revealed no gross morphological damage in intestinal tissues from stressed rats. This implies that the results reported relate to physiological abnormalities rather than to gross tissue injury as a result of stress or cold or that physiological changes precede more obvious injury. Additional experiments are justified to examine the longer term abnormalities in these animals and the mechanism by which such changes occur.

Elevation of circulating glucocorticoids is recognized as a classic response to stress [31]; however, its level does not necessarily reflect the amount of stress perceived in every situation. Plasma corticosterone was increased in stressed rats compared with controls, and a mild increase was caused by fasting. Corticosteroids have been reported to affect some aspects of gastrointestinal mucosal function. Methylprednisolone for 3 days increased jejunal and ileal $\text{Na}^+ - \text{K}^+$ -adenosinetriphosphatase activity, leading to increased absorption of Na^+ [8]. However, another study reported that methylprednisolone increased guanylate cyclase activity in the jejunum and ileum 6 h after administration, resulting in increased Cl^- secretion [17]. Those effects occurred in response to higher doses of steroid than the circulating levels documented in our stressed rats. Therefore, it is improbable that the effects reported in this article were due directly to the increased levels of corticosterone recorded in stressed rats. In addition, even though cold enhanced the changes in rat intestine caused by restraint

(a common finding in stress-induced gastric ulceration), it had no significant enhancing effect on the level of corticosterone in the blood. However, studies in adrenalectomized rats are needed to test directly the role of corticosteroid hormones in the stress responses we documented.

In summary, we have characterized two important intestinal physiological alterations in rats exposed to acute stressors: stimulation of Cl⁻ secretion and increased permeability to ions and larger molecules. In addition, we have provided evidence for neural changes that may indicate involvement of nerves in these abnormalities. The model we have presented will allow the factors involved to be identified and may help explain the role of psychological factors in intestinal dysfunction.

The authors acknowledge the expert technical assistance of M. Benjamin. This research was supported by grants from the Crohn's Colitis Foundation of Canada, the Medical Research Council of Canada, and the National Institutes of Health (NS-29536).

2.5 REFERENCES

1. Almy, T. P., L. E. Hinkle, B. Berk, and F. Kern. Alterations in colonic function in man under stress. III. Experimental production of sigmoid spasm in patients with spastic constipation. *Gastroenterology* 12: 437-449, 1949.
2. Almy, T. P., F. Kern, and M. Tulin. Alterations in colonic function in man under stress. II. Experimental production of sigmoid spasm in healthy persons. *Gastroenterology* 12: 425-436, 1949.
3. Barclay, G. R., and L. A. Turnberg. Effect of psychological stress on salt and water transport in the human jejunum. *Gastroenterology* 93: 91-97, 1987.
4. Barclay, G. R., and L. A. Turnberg. Effect of cold-induced pain on salt and water transport in the human jejunum. *Gastroenterology* 94: 994-998, 1988.
5. Barclay, G. R., and L. A. Turnberg. Effect of moderate exercise on salt and water transport in the human jejunum. *Gut* 29: 816-820, 1988.
6. Beaumont, W. Experiments and Observations on the Gastric Juices and the Physiology of Digestion. Edinburgh, UK: Neill, 1838.
7. Brown, D. R., A. M. Parsons, and M. O'Grady. Substance P produces sodium and bicarbonate secretion in porcine jejunal mucosa through an action on enteric neurons. *J. Pharmacol. Exp. Ther.* 261: 1206-1212, 1992.
8. Charney, A. N., M.D. Kinsey, L. Myers, R. A. Giannella, and R.E. Gots. Na⁺-K⁺-activated adenosine triphosphatase and intestinal electrolyte transport: effect of adrenal steroids. *J. Clin. Invest.* 56: 653-660, 1975.
9. Cooke, H. Neural and humoral regulation of small intestinal electrolyte transport. In: *Physiology of the Gastrointestinal Tract*, edited by L. R. Johnson. New York: Raven, 1987, chapt. 47, pg. 1307-1350.
10. Duffy, L. C., M. A. Zielezny, J. R. Marshall, T. E. Byers, M. M. Weiser, J. F. Phillips, B. M. Calkins, P. L. Ogra, and S. Graham. Relevance of major stress events as an indicator of disease activity prevalence in inflammatory bowel disease. *Behav. Med.* 17: 101-110, 1991.
11. Empey, L. R., and R. N. Fedorak. Effect of misoprostol in preventing stress-induced intestinal fluid secretion in rats. *Prostaglandins Leukotrienes Essent. Fatty Acids* 38: 43-48, 1989.

12. Furnell, J. R., and P. V. Dutton. Alleviation of toddlers diarrhoea by environmental management. *J. Psychosom. Res.* 30: 282-288, 1986.
13. Glavin, G. B. Restraint ulcer: history, current research and future implications. *Brain Res. Bull.* 5: 51-58, 1980.
14. Hollander, D. D. Ricketts, and C. A. R. Boyd. The importance of "probe" molecular geometry in determining intestinal permeability. *Can. J. Gastroenterol.* 2: 35-38, 1988.
15. Javed, N. H., and H. J. Cooke. Acetylcholine release from colonic submucous neurons associated with chloride secretion in the guinea pig. *Am. J. Physiol.* 262 (Gastrointest. Liver Physiol. 25): G131-G136, 1992.
16. Lucas, C. E., C. Sugawa, J. Riddle, F. Rector, B. Rosenberg, and A. J. Walt. Natural history and surgical dilemma of "stress" gastric bleeding. *Arch. Surg.* 102: 266-272, 1970.
17. Marnane, W. G., Y. H. Tai, R. A. Decker, E. C. Boedeker, A. N. Charney, and M. Donowitz. Methylprednisolone stimulation of guanylate cyclase activity in rat small intestinal mucosa: possible role in electrolyte transport. *Gastroenterology* 81: 90-100, 1981.
18. McCrae, S., K. Younger, D. G. Thompson, and D. L. Wingate. Sustained mental stress alters human jejunal motor activity. *Gut* 23: 404-409, 1982.
19. Narducci, F., W. J. Snape, W. M. Battle, R. L. London, and S. Cohen. Increased colonic motility during exposure to a stressful situation. *Dig. Dis. Sci.* 30: 40-44, 1985.
20. Pare, W. P. Strain, age, but not gender, influence ulcer severity induced by water-restraint stress. *Physiol. Behav.* 45: 627-632, 1989.
21. Pare, W. P. Stress ulcer susceptibility and depression in Wistar Kyoto (WKY) rats. *Physiol. Behav.* 46: 993-998, 1989.
22. Perdue, M. H., M. Chung, and D. G. Gall. The effect of intestinal anaphylaxis on gut function in the rat. *Gastroenterology* 86: 391-397, 1984.
23. Perdue, M. H., and J. S. Davison. Response of jejunal mucosa to electrical transmural stimulation and two neurotoxins. *Am. J. Physiol.* 251 (Gastrointest. Liver Physiol. 14): G642-G648, 1986.

24. Perdue, M. H., R. Gailbraith, and J. S. Davison. Altered regulation of intestinal ion transport by enteric nerves in diabetic rats. *Am. J. Physiol.* 251 (Gastrointest. Liver Physiol. 14): G444- G449, 1988.
25. Perdue, M. H., and D. G. Gall. Intestinal anaphylaxis in the rat: jejunal response to *in vitro* antigen exposure. *Am. J. Physiol.* 250 (Gastrointest. Liver Physiol. 13): G427- G431, 1986.
26. Phillips, T. E., T. L. Phillips, and M. R. Neutra. Macromolecules can pass through occluding junctions of rat ileal epithelium during cholinergic stimulation. *Cell Tissue Res.* 247: 547-554, 1987.
27. Ramage, J. K., A. Stanis, R. Scicchitano, R. H. Hunt, and M. H. Perdue. Effect of immunological reactions on rat intestinal epithelium. Correlation of increased gut permeability to chromium 51-labelled ethylenediaminetetraacetic acid and ovalbumin during inflammation and anaphylaxis in the rat. *Gastroenterology* 94: 1368-1375, 1988.
28. Ray, A., R. M. Sullivan, and P. G. Henke. Adrenergic modulation of gastric stress pathology in rats: a cholinergic link. *J. Auton. Nerv. Syst.* 20: 265-268, 1987.
29. Selye, H. A syndrome produced by diverse nocuous agents. *Nature Lond.* 138: 32, 1936.
30. Selye, H. The general adaptation syndrome and the diseases of adaptation. *J. Clin. Endocrinol.* 6: 117-230, 1946.
31. Selye, H. Stress. The Physiology and Pathophysiology of Exposure to Stress. Montreal, Canada: Acta, 1950.
32. Travis, S., and I. Menzies. Intestinal permeability-functional assessment and significance. *Clin. Sci. Lond.* 82: 471-488, 1992.
33. Wang, J.-Y., and L. R. Johnson. Induction of gastric and duodenal mucosal ornithine decarboxylase during stress. *Am. J. Physiol.* 257 (Gastrointest. Liver Physiol. 20): G259-G265, 1989.

NOTE TO USERS

**Page(s) missing in number only; text follows.
Microfilmed as received.**

57

This reproduction is the best copy available.

UMI

The following work, my second publication, addresses a putative mechanism mediating the effects of acute stress on intestinal (jejunal) epithelial physiology. The experiments in this paper use a very similar model to the previous paper, but we inhibit the epithelial stress responses by pretreating rats with a drug that blocks acetylcholine activity. The results also indicate a physiological difference in the cholinergic activity in the rats I use for these experiments versus their parent strain (which they were originally bred from) which suggests why these rats are stress susceptible.

Chapter 3

Cholinergic nerves mediate stress-induced intestinal transport abnormalities in Wistar Kyoto rats.

P.R. Saunders, N.P.M. Hanssen and M.H. Perdue.

American Journal of Physiology, 273: G486-G490, 1997.

Copyright held by American Physiological Society

NOTE TO USERS

**Page(s) missing in number only; text follows.
Microfilmed as received.**

60

This reproduction is the best copy available.

UMI

ABSTRACT

We have previously reported that acute stress alters intestinal transport physiology in Wistar Kyoto rats, a stress susceptible strain. In this study, we tested the hypothesis that the abnormalities in these rats are due to cholinergic mechanisms. Atropine or saline treated rats were exposed to acute restraint stress and subsequently electrophysiological parameters of excised jejunal segments were assessed in Ussing chambers. Compared to the parent Wistar rat strain, Wistar Kyoto rats demonstrated significantly greater stress-induced changes in ion secretion and permeability. The activity of cholinesterase in intestinal mucosal homogenates was significantly less in Wistar Kyoto than Wistar rats. Atropine pretreatment of rats prior to stress corrected the epithelial pathophysiology. Our results suggest that stress stimulated the release of acetylcholine resulting in altered epithelial function in these genetically predisposed rats.

3.1 INTRODUCTION

Since Selye first described gastric ulceration as one of the three general responses to stress [24], numerous experimental studies have confirmed the relationship between psychological stress and gastric ulceration [12]. More recent reports have demonstrated stress-induced abnormalities of gastrointestinal motility [6, 18]. However, the effect of stress on the intestinal mucosa has received relatively little attention. A few studies have reported

stress-induced intestinal fluid secretion in humans and rats [3,9], but did not explore the neural pathways or neurotransmitters involved.

The transport function of intestinal epithelium is generally accepted to be regulated by intrinsic intestinal nerves [7]. Signals from the central nervous system to the gut mucosa can be communicated via autonomic extrinsic nerves or their connections to intrinsic nerves in the plexus regions of the gut wall [10]. Electrical stimulation of the vagus in anaesthetized ferrets was shown to increase the overall potential difference (PD) across the jejunum as well as initiate cyclical fluctuations in PD [14]. Stimulation in the rat brain stem, specifically the dorsal motor nucleus which is in series with the vagus, significantly reduced water absorption in the ileum [17]. However, much less is known about the regulation of the epithelial barrier function, including the neural circuits that may be involved.

We previously reported [23] that acute stress caused intestinal mucosal pathophysiology in Wistar Kyoto rats. Following exposure of rats to restraint stress with or without cold, jejunal preparations demonstrated an increased baseline short-circuit current (I_{sc}) due to net Cl⁻ ion secretion, an impaired secretory response to electrical transmural stimulation of enteric nerves, and increased epithelial permeability to ions and inert radiolabelled probes. Here, we tested the hypothesis that cholinergic nerves are involved in transducing stress signals from the central nervous system to the gut for several reasons: 1) Wistar Kyoto rats have been reported to have low levels of circulating cholinesterase activity [16] and therefore may have increased sensitivity to acetylcholine; 2) cholinergic nerves play a major role in regulating epithelial ion transport via muscarinic receptors on epithelial cells [7]; and 3) cholinergic nerves have been shown to play a critical role in mediating other stress-

induced gastrointestinal pathology such as gastric ulceration [11]. Therefore, in these studies we compared stress induced changes in intestinal physiology in Wistar Kyoto rats versus those in the parent Wistar strain and examined the role of cholinergic nerves and muscarinic or nicotinic receptors in mediating the responses.

3.2 METHODS

3.2.1 Animals

These experiments were approved by the Animal Care Committee at McMaster University. The procedures used were similar to those reported in our earlier study [23]. Briefly, adult male rats (140-160g) of the Wistar Kyoto strain and the parent Wistar strain (Charles River, St. Constant, Quebec, Canada) were maintained on a normal light/dark cycle and provided with food and water ad libitum. Rats were handled daily by the same investigator for 2 weeks and were fasted overnight (14 h) before the experiment. Rats in the stress group were placed in a leucite adjustable restraining device (6 x 21.5 cm) for 2 h in a room with an ambient temperature of 8°C, followed by 2 h of free movement in their home cage at 22°C (please see Appendix B for Time Course data indicating the appropriate rest period following 2h of stress). Control rats were treated similarly but left in their home cage before study. The rats were then anaesthetized with im urethane (Sigma Chemical Co., St. Louis, MO) and maintained at 37°C via a rectal probe and thermally controlled heating pad for ~ 30 min until a laparotomy was performed. A 20 cm segment of jejunum, beginning 5 cm distal to the ligament of Treitz, was removed and mucosa was scraped from the most distal 5 cm and stored at -70°C for later determination of cholinesterase activity. The

remaining segment was placed in 37°C oxygenated Krebs buffer for Ussing chamber studies.

3.2.2 Ussing Chamber Studies

The segment was stripped of external muscle, opened along its mesenteric border and cut into 4 pieces that were mounted in Ussing chambers (opening of 0.6 cm²). Tissues were bathed in 37°C oxygenated Krebs buffer (10 ml each side) containing (in mM): 115 NaCl, 1.25 CaCl₂, 1.2 MgCl₂, 2.0 KH₂PO₄, 25 NaHCO₃ @ pH 7.35±0.02. The serosal buffer included 10 mM glucose as an energy source, osmotically balanced by 10 mM mannitol in the mucosal (luminal) buffer. The chambers contained agar-salt bridges to monitor PD and introduce Isc via an automatic voltage clamp (W-P Instruments, Narco Scientific, Downsview, Canada). Conductance (G) was calculated from Ohm's law using PD and Isc values. Baseline values for Isc and G were recorded at equilibrium, 20 min after mounting the tissue in Ussing chambers. In some experiments, the Isc response to acetylcholine (5×10⁻⁵M; Sigma) added to the serosal compartment was determined.

3.2.3 Permeability

The inert probe, ⁵¹Cr-EDTA (6 µCi/ml) (Radiopharmacy, Chedoke-McMaster Hospital, Hamilton, Canada), was added to the mucosal buffer of the Ussing chambers, balanced by an equivalent concentration of unlabelled Cr-EDTA in the serosal buffer. Four 1 ml samples were taken from the "cold" serosal buffer at 20 min intervals after equilibrium (30 to 90 min period), while duplicate 0.05 ml samples were obtained from the "hot" mucosal buffer at the beginning and end of the experiment to calculate specific activity. The

radioactivity of ^{51}Cr -EDTA was measured in a gamma counter. Transepithelial fluxes were calculated by standard formulae and were expressed as $\text{nmol}/\text{cm}^2/\text{h}$.

3.2.4 Cholinergic Receptor Antagonism

Thirty min prior to being placed in the restraining device, Wistar Kyoto rats were injected ip with either atropine sulphate (general muscarinic antagonist), atropine methyl nitrate (a peripheral muscarinic antagonist that does not cross the blood brain barrier), or hexamethonium (nicotinic antagonist) (all drugs from Sigma). A dose of 10 mg/kg was chosen based on pilot studies with atropine sulphate and the consideration that Wistar Kyoto rats might have increased cholinergic activity/sensitivity and require a somewhat higher dose than that usually employed (1 mg/kg [12]). Control rats were injected with an equivalent volume of saline.

To determine if on-going release of acetylcholine in the intestine was directly responsible for stress-induced epithelial transport changes, atropine sulphate was added to the serosal side of Ussing chambers containing tissues from untreated stressed rats. A concentration of 10^{-5} M atropine was chosen since it blocked the ΔIsc to acetylcholine or to the muscarinic agonist, bethanechol (both at 10^{-4} M), added to the serosal side of tissues from control rats. Tissues from stressed rats were paired such that 2 tissues received atropine and 2 tissues received vehicle (buffer). Values for baseline Isc , conductance and flux of ^{51}Cr -EDTA in atropine treated tissues were expressed as percent of values in untreated paired tissues.

3.2.5 Cholinesterase Activity

We measured total cholinesterase activity in mucosal homogenates rather than the activity of acetylcholinesterase since both acetyl and butyrylcholinesterase metabolize acetylcholine in rat intestine [25]. The mucosal samples were homogenized in 2 ml phosphate buffer and the supernatant was taken for assay (method modified from Ellman [8]). The following, in percentage (w/v) were added to phosphate buffer (pH 8.0): 96.6% Ellman's reagent; 36.6% NaHCO₃; 26.5% acetylthiocholine iodide (Sigma); and 500µl of sample. The rate of change in absorbance (405 nm) over 60 sec was used to calculate the total cholinesterase activity in the sample. Units of activity are indicated as µmol/min and are expressed per mg of mucosal wet weight.

3.2.6 Statistics

One-way ANOVA was used with Neuman Keul's as a subsequent multiple comparison test, as well as two-way ANOVA where appropriate. Single comparisons were performed by Student's t-test [15]. Statistical significance was defined at $p < 0.05$. Values for individual tissues from each rat were averaged before conducting statistics on values for rat groups ($n =$ number of rats). Results are expressed as mean \pm SEM.

3.3 RESULTS

3.3.1 Intestinal Parameters in Control and Stressed Wistar and Wistar Kyoto Rats

Table 3.1 shows that jejunum from control Wistar Kyoto rats had a significantly higher baseline Isc than jejunum from Wistar rats. Stress caused a significant increase of Isc in

intestine from both strains. However, the magnitude of the change was significantly ($p=0.008$) greater, increased ~ 2 fold ($\Delta 28$ versus $\Delta 15$, $\mu\text{A}/\text{cm}^2/\text{h}$), in tissue from Wistar Kyoto rats compared with Wistar rats. Values for baseline conductance were not significantly different in jejunal tissues from the two rat strains. However, again stress caused a significantly ($p<0.005$) greater change in conductance, increased > 3 fold ($\Delta 28$ versus $\Delta 8$, mS/cm^2) in jejunum from Wistar Kyoto rats compared with Wistar rats.

TABLE 3.1 Transport Parameters in Jejunum from Wistar and Wistar Kyoto Rats

	Isc ($\mu\text{A}/\text{cm}^2$)		G (mS/cm^2)	
	control	stress	control	stress
Wistar	24.0 ± 3.8	39.5 ± 7.0^b	32.6 ± 1.7	40.5 ± 2.7^b
WKY	31.2 ± 1.6^a	59.4 ± 4.3^{ab}	35.4 ± 1.3	63.8 ± 5.1^{ab}

Jejunal segments from control and stressed Wistar and Wistar Kyoto (WKY) rats were mounted in Ussing chambers. Baseline short-circuit current (Isc) and conductance (G) were recorded at equilibrium (20 min after mounting). Two-way ANOVA indicated two significant main effects for the factors stress and strain for Isc ($p<0.001$ and $p=0.008$ respectively) with stressed Wistar Kyoto rats having the highest response. There was a significant two-way interaction between the factors stress and strain for the G measures ($p=0.005$) with Wistar Kyoto rats responding to stress more intensely than Wistar rats. Values represent means \pm SEM; $n = 5-6$ for all groups; ^a indicates significant difference from value in Wistar rats, ^b indicates significant difference from value in control rats of same strain.

We conducted studies to determine if increased cholinergic sensitivity might explain the greater stress-induced changes in intestinal physiology of Wistar Kyoto versus Wistar rats. Figure 3.1 demonstrates that tissue from Wistar Kyoto rats had a significantly greater ($p=0.019$) Isc response to acetylcholine than Wistar rats (Δ Isc to $5 \times 10^{-5}\text{M}$ acetylcholine of 72 ± 8 versus $49 \pm 5 \mu\text{A}/\text{cm}^2$). Decreased breakdown of acetylcholine in intestinal mucosa

might explain such changes. Therefore, we analysed mucosal scrapings from intestinal segments of the two rat strains for cholinesterase activity. Figure 3.1 shows that Wistar Kyoto rats had significantly ($p=0.004$) less mucosal cholinesterase activity compared with Wistar rats (2.4 ± 0.1 versus 4.4 ± 0.4 units/mg).

3.3.2 Effect of Cholinergic Antagonism on Stress-Induced Intestinal Pathophysiology

To determine if cholinergic factors play a critical role in the stress-induced gut pathophysiology, we examined the effect of antagonizing cholinergic muscarinic receptors. Atropine sulphate treatment of control Wistar Kyoto rats had no effect on any baseline intestinal transport parameter. As before, stress caused a significant increase ($p<0.001$) in the baseline *I*_{sc} of intestinal tissues. The stress-induced increased *I*_{sc} was inhibited ($p<0.05$) in rats treated with atropine sulfate, resulting in similar values in tissues from treated stressed rats compared with controls (Figure 3.2). In addition, stress increased epithelial permeability as reflected by the significantly ($p<0.001$) elevated conductance (Figure 3.3A), and the significantly ($p=0.002$) increased flux value for the probe, ⁵¹Cr-EDTA (Figure 3.3B). Again, pretreatment of rats with atropine sulfate abolished these stress-induced changes. However, atropine sulphate added directly to the chambers (5×10^{-5} M) containing intestine from stressed rats, did not normalize the stress-induced abnormalities (values for tissues with atropine expressed as percent of values for paired tissues without atropine: *I*_{sc} $105 \pm 13\%$; conductance $103 \pm 7\%$; flux of ⁵¹Cr-EDTA $92 \pm 5\%$; $n = 8$ rats). This suggests that blocking muscarinic receptors following stress is not effective in reversing gut abnormalities.

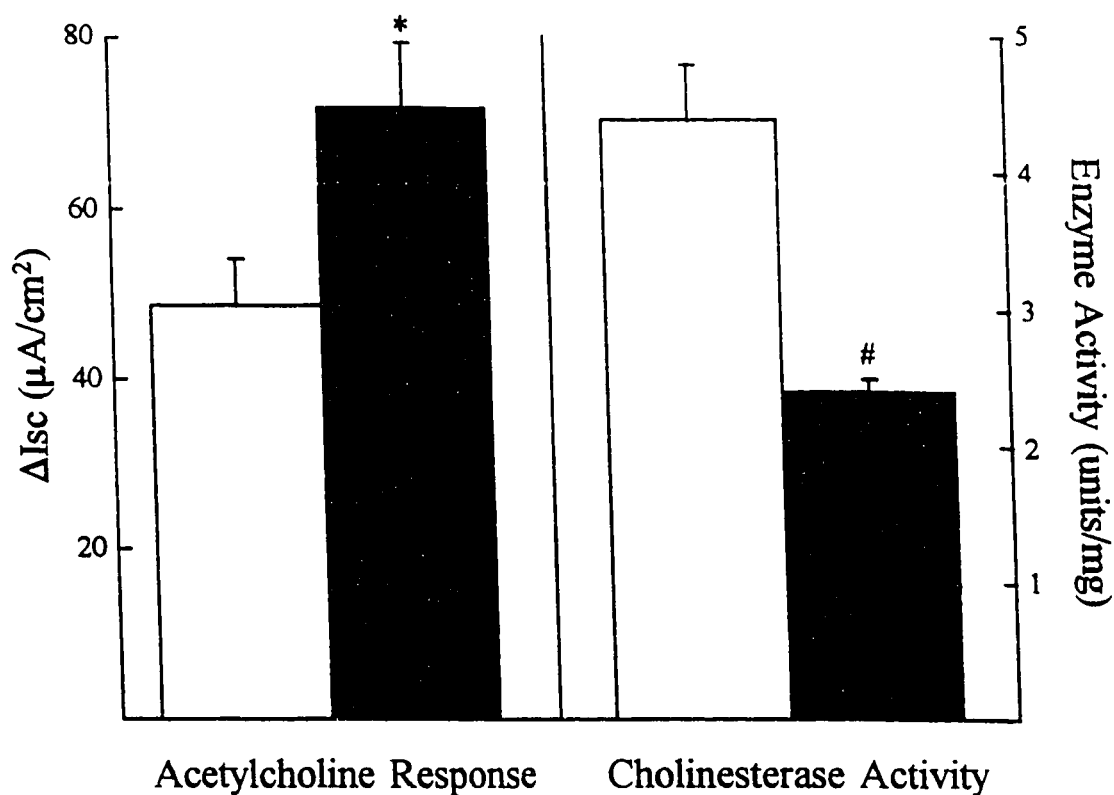


Figure 3.1 Response to acetylcholine and cholinesterase activity in jejunum from Wistar and Wistar Kyoto rats. The left panel is the change in short-circuit current (ΔI_{sc}) above baseline following challenge of the tissues with $5 \times 10^{-5} M$ acetylcholine. The response of the tissue from Wistar Kyoto rats (solid bars) was significantly greater (* $p=0.019$) than the response of tissue from Wistar rats (hatched bars). The right panel is the cholinesterase activity (units/mg wet weight) in jejunal mucosal scrapings. Student's t-test indicated that Wistar Kyoto rats had significantly less total cholinesterase activity in their mucosa compared to Wistar rats (# $p=0.004$). Values represent means \pm SEM; $n = 7-8$ rats/group.

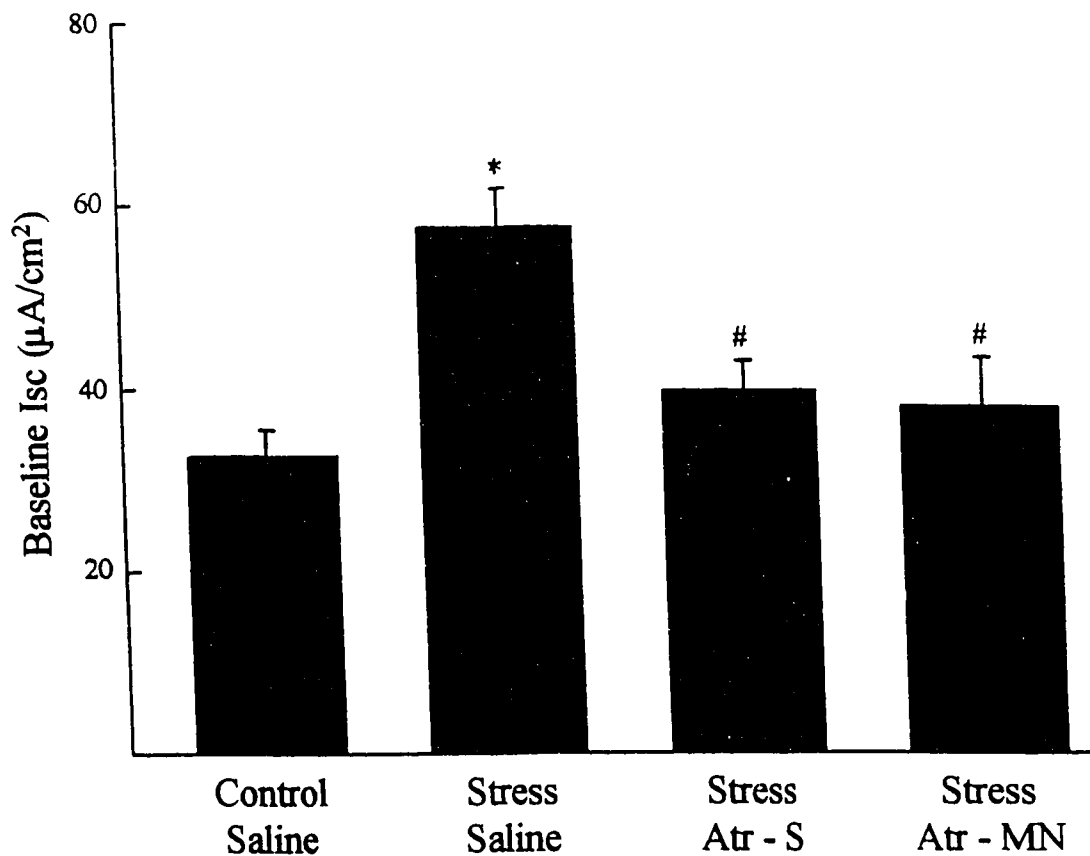


Figure 3.2 Baseline short-circuit current (Isc) of jejunum from control or stressed Wistar Kyoto rats. Rats were injected 30 min prior to stress with either saline, atropine sulfate (Atr-S), or atropine methyl nitrate (Atr-MN). Isc was recorded at equilibrium (20 min after mounting tissues in Ussing chambers). One-way ANOVA indicated a significant difference for baseline Isc ($p < 0.001$); * indicates significant difference ($p < 0.05$) compared to control/saline, # indicates significant difference ($p < 0.05$) compared to stress/saline by Neuman Keul's multiple comparison test. Values represent means \pm SEM; $n = 7-8$ rats/group.

To determine the site of action for the inhibitory action of atropine, the effects of the peripherally acting antagonist, atropine methyl nitrate that does not cross the blood-brain barrier, were evaluated. Results were similar to those of atropine sulfate, including inhibition of the stress-induced increased baseline I_{sc}, conductance and fluxes of ⁵¹Cr-EDTA (Figures 3.2 and 3.3).

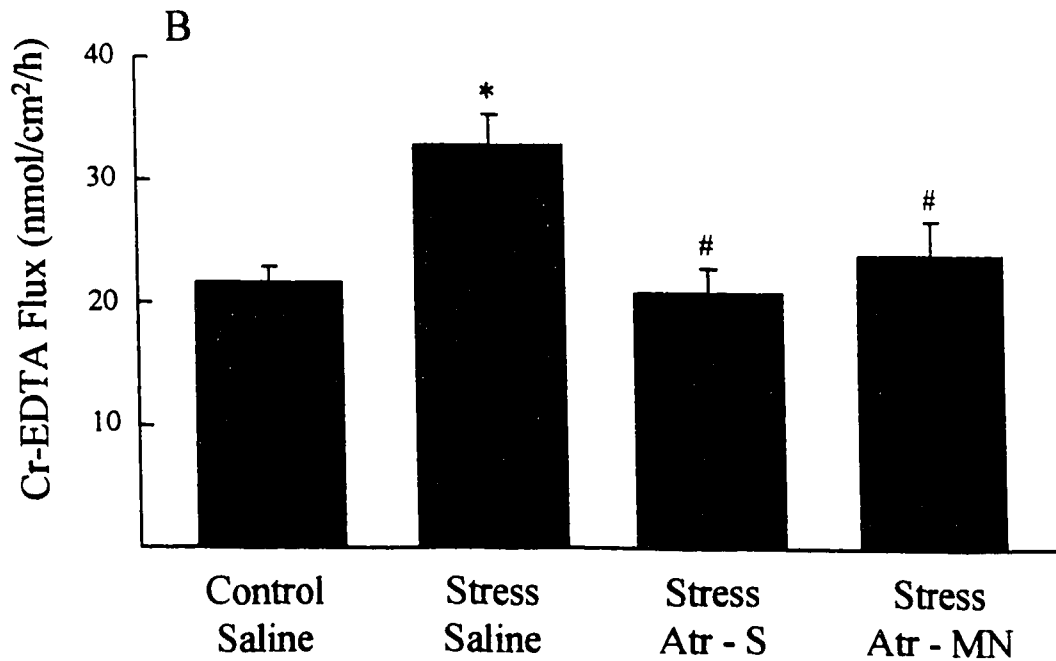
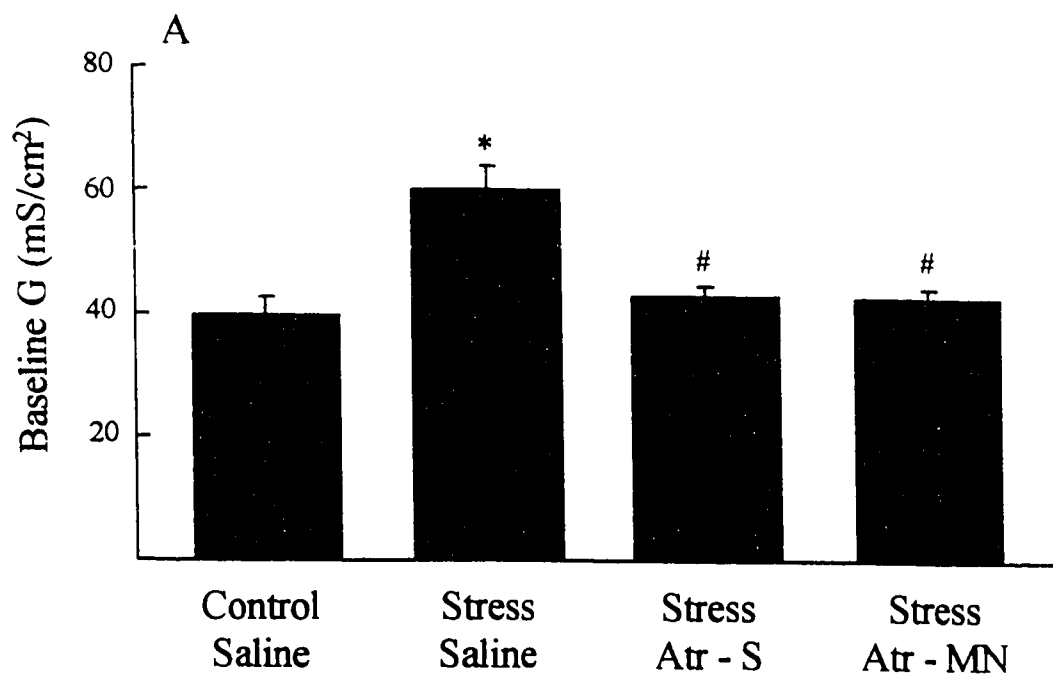
Antagonism of nicotinic receptors had no significant beneficial effect. The stress-induced intestinal abnormalities were unchanged by pretreatment of rats with hexamethonium. Values were $52 \pm 4 \mu\text{A}/\text{cm}^2/\text{h}$ for I_{sc} and $55 \pm 7 \text{mS}/\text{cm}^2$ for conductance ($n = 4$ rats), not significantly different from those of untreated stressed rats. Flux experiments were not conducted.

3.4 DISCUSSION

Our results demonstrate that the intestinal transport responses to stress were exaggerated in Wistar Kyoto rats. The magnitude of the stress-induced changes in baseline I_{sc} and conductance values in jejunal tissues were significantly greater in Wistar Kyoto rats compared with Wistar rats, supporting the concept that Wistar Kyoto rats are more susceptible to stress than Wistar rats. This is consistent with other studies reporting that Wistar Kyoto rats are a stress susceptible strain [13,21].

Wistar Kyoto rats were previously reported to have significantly lower (50%) cholinesterase activity in serum compared with Sprague Dawley rats [16]. We showed that Wistar Kyoto rats also have significantly lower cholinesterase activity in intestinal mucosa compared with Wistar rats, the parent strain from which the Wistar Kyoto rats were derived.

Figure 3.3 Permeability parameters of jejunum from control and stressed Wistar Kyoto rats. A) Baseline conductance (G) of tissue recorded at equilibrium (20 min after mounting tissues in Ussing chambers). B) The mucosal to serosal flux of Cr-EDTA across tissues over a 60 min period. Rats were injected 30 min prior to stress with either saline, atropine sulfate (Atr-S), or atropine methyl nitrate (Atr-MN). One-way ANOVA indicated a significant difference for baseline conductance and Cr-EDTA flux ($p < 0.001$ and $p = 0.002$ respectively). * significant difference ($p < 0.05$) compared to control/saline; # significant difference ($p < 0.05$) compared to stress/saline by Neuman Keul's multiple comparison test. Values represent means \pm SEM; $n = 7-8$ rats/group.



The decreased ability to degrade acetylcholine in the mucosa may cause exaggerated cholinergic responses and may account for the enhanced intestinal responses to stress we observed in Wistar Kyoto rats. This concept is further supported by the fact that Isc responses to challenge with exogenous acetylcholine were greater in intestinal jejunum from Wistar Kyoto rats compared with Wistar rats. Wistar Kyoto rats were initially bred as the control strain for the Spontaneously Hypertensive Rat (SHR) strain. Subsequently, it was identified that Wistar Kyoto rats have an increased cholinergic sensitivity [16]. Evidence that hypercholinergic activity can play a pivotal role in an animal's response to stress also comes from studies using rats of the Flinders Sensitive Line. These rats were specifically bred to be sensitive to cholinergic stimulation [20] and were later shown to be more susceptible to stress than rats of the control inbred Flinders Resistant Line [22].

Our study demonstrated that the changes in stress-induced epithelial physiology of the jejunum were mediated by acetylcholine acting on muscarinic receptors. Atropine sulfate had no effect on baseline intestinal parameters in control Wistar Kyoto rats that had been handled daily for 2 weeks prior to the experiment, suggesting that on-going release of acetylcholine is not involved in maintaining basal transport physiology in this rat strain. However, atropine sulphate inhibited the intestinal responses to stress in these rats, including the increased baseline Isc (previously shown to be due to chloride ion secretion [23]), the increased permeability to ions (measured by conductance) and to the larger probe molecule $^{51}\text{Cr-EDTA}$ (measured by flux). To determine if acetylcholine was acting peripherally or centrally [4], we pretreated rats with atropine methyl nitrate, a quaternary salt of atropine that does not cross the blood brain barrier [5]. Atropine methyl nitrate also blocked the stress-induced increase

in baseline Isc, conductance and the increased flux of $^{51}\text{Cr-EDTA}$. Collectively, these findings suggest that acetylcholine mediated the intestinal transport abnormalities via muscarinic receptors located in the gastrointestinal tract. The fact that hexamethonium was ineffective in inhibiting the stress-induced gut abnormalities implies that nicotinic receptors were not involved in the pathway by which acetylcholine mediates the intestinal responses to stress.

Other investigators examining gastric ulceration and altered colonic motility following water stress showed that a significant proportion of the message was transmitted to the viscera via several branches of the vagus [19,27]. In our model, atropine completely blocked the stress-induced epithelial pathophysiology in the jejunal mucosa. Therefore, it seems reasonable to postulate that parasympathetic nerves, in particular vagal branches, are the pathway by which signals are transmitted from the central nervous system to the small intestine. Current studies are exploring the role of the vagus and extrinsic nerves in stress-induced changes in intestinal mucosal function.

Acetylcholine and cholinergic nerves may be directly responsible for the epithelial responses to stress, although suggestive evidence indicates there may also be indirect effects via other mucosal cells. In our study, atropine added directly to the chambers did not alter the stress-induced intestinal abnormalities. This may have been because the atropine that had been administered *in vivo* 4.5 h previously was no longer effective or had been washed away by the circulating buffer in the Ussing chambers. This finding may also suggest that the release of acetylcholine is only one of several endogenous transmitters/mediators involved in the stress-induced pathophysiology. Other studies have reported that vagal stimulation can cause histamine release from mast cells in rat ileum [2]. In addition, acute stress released substance

P from guinea pig airways and chronic stress altered tissue levels of substance P [1]. Substance P is known to activate mucosal mast cells [26]. Our finding that the intestinal secretory responses were paralleled by a significant increase in paracellular permeability is consistent with nerve and/or inflammatory cell regulation of tight junction permeability. Studies in progress in our laboratory support this concept. However, it is clear that cholinergic nerves are critical in the pathway since atropine pretreatment of rats completely abrogated the stress-induced pathophysiology.

In summary, this study contributes new information on the role of psychological factors such as stress in intestinal mucosal dysfunction and provides evidence for cholinergic involvement in these abnormalities. In addition, we have demonstrated that susceptibility to stress may be due to changes at the level of the nervous system rather than the hypothalamic-pituitary-adrenal axis. Stress-induced increases in epithelial permeability that allows uptake of noxious material from the gut lumen may be important in triggering or perpetuating intestinal inflammation.

The authors acknowledge the expert technical assistance of Michelle Benjamin, the contribution of Dihan Chandraratne and the help of Dr. M. Blennerhassett with the cholinesterase assay. This research was supported by the Medical Research Council of Canada.

3.5 REFERENCES

1. Abe, T., S. Yoshihara, T. Ando and T. Ichimura. The influence of frequency of cold-water bathing on the substance P content of the airways in the guinea pigs. *Regulatory Peptides* 41: S29, 1992.
2. Bani-Sacchi, T., M. Barattini, S. Bianchi, P. Blandina, S. Brunelleschi, R. Fantozzi, P.F. Mannaioni and E. Masini. The release of histamine by parasympathetic stimulation in guinea-pig auricle and rat ileum. *J. Physiol.* 371: 29-43, 1986.
3. Barclay, G.R. and L.A. Turnberg. Effect of psychological stress on salt and water transport in the human jejunum. *Gastroenterology* 93: 91-7, 1987.
4. Bhargava, K.P., M. Daas, G.P. Gupta and M.B. Gupta. Study of central neurotransmitters in stress-induced gastric ulceration in albino rats. *Br. J. Pharmacol.* 68: 765-72, 1980.
5. Brown, J.H. Atropine, Scopolamine, and Related Antimuscarinic Drugs. In Goodman and Gilman's the Pharmacological Basis of Therapeutics, 8th edition. A.G. Gilman, T.W. Rall, A.S. Nies and P. Taylor (Eds). New York, Pergamon Press. 1990, pp 150-65.
6. Bueno, L., S.M. Collins and J.L. Junien. Stress and Digestive Motility. London, John Libby Publishers. 1989.
7. Cooke, H.J. and R.A. Reddix. Neural regulation of intestinal electrolyte transport. In Physiology of the Gastrointestinal Tract, L.R. Johnson (Ed.). New York, Raven Press. 1994, pp 2083-132.
8. Ellman, G.L., K.D. Courtney, V. Andres, Jr. and R.M. Featherstone. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7: 88-95, 1961.
9. Empey, L.R. and R.N. Fedorak. Effect of misoprostol in preventing stress-induced intestinal fluid secretion in rats. *Prostaglandins Leukotrienes Essent. Fatty Acids.* 38: 43-8, 1989.
10. Furness, J.B. and M. Costa. The Enteric Nervous System. New York, Churchill Livingstone. 1987.

11. Gatón, J., F. Fernández de la Gándrara and A. Velasco. The role of the neurotransmitters acetylcholine and noradrenaline in the pathogenesis of stress ulcers. *Comp. Biochem. Physiol.* 106C: 125-9, 1993.
12. Glavin, G.B. Restraint ulcer: history, current research and future implications. *Brain Res. Bull.* 5: 51-8, 1980.
13. Glowa, J.R. and C.T. Hansen. Differences in response to an acoustic startle stimulus among forty-six rat strains. *Behav. Genetics.* 24: 79-84, 1994.
14. Greenwood, B. and J.S. Davison. Role of extrinsic and intrinsic nerves in the relationship between intestinal motility and the transmural potential difference in the anaesthetized ferret. *Gastroenterology* 89: 1286-92 1985.
15. Hopkins, K.D., G.V. Glass and B.R. Hopkins. Basic Statistics for the Behavioral Sciences, Second Edition. Englewood Cliffs, N.J., Prentice-Hall, Inc. 1987.
16. Lim, D.K., I.B. Hoskins, R.W. Rockhold and I.K. Ho. Comparative studies of muscarinic and dopamine receptors in three strains of rat. *Eur. J. Pharmacol.* 165: 279-87, 1989.
17. Martin, K., T.H. Kong, W. Renehan, A. Schurr, W. Dong, X. Zhang and R. Fogel. Identification and function of brain stem neurons regulating rat ileal water absorption. *Am. J. Physiol.* 257: G266-73, 1989.
18. McCrae, S., K. Younger, D.G. Thompson and D.L. Wingate. Sustained mental stress alters human jejunal motor activity. *Gut.* 23: 404-9, 1982.
19. Monnikes, H., B.G. Schmidt, H.E. Raybould and Y. Taché. CRF in the paraventricular nucleus mediates gastric and colonic motor response to restraint stress. *Am. J. Physiol.* 262: G137-43, 1992.
20. Overstreet, D.H. Selective breeding for increased cholinergic function: development of a new animal model of depression. *Biol. Psych.* 21: 49-58, 1986.
21. Pare, W.P. Stress ulcer susceptibility and depression in Wistar Kyoto (WKY) rats. *Physiol. Behav.* 46: 993-8, 1989.
22. Pucilowski, O., D.H. Overstreet, A.H. Rezvani and D.S. Janowsky. Chronic mild stress-induced anhedonia: greater effect in a genetic rat model of depression. *Physiol. Behav.* 54: 1215-20, 1993.

23. Saunders, P.R., U. Kosecka, D.M. McKay and M.H. Perdue. Acute stressors stimulate ion secretion and increase epithelial permeability in rat intestine. *Am. J. Physiol.* 267: G794-9, 1994.
24. Selye, H. A syndrome produced by diverse nocuous agents. *Nature.* 138: 32, 1936.
25. Sine, J-P., R. Ferrand and B. Colas. Acetylcholinesterase and butyrylcholinesterase in the gut mucosal cells of various mammal species: distribution along the intestine and molecular forms. *Comp. Biochem. Physiol.* 91C: 597-602, 1988.
26. Wang, L., A.M. Stanisiz, B.K. Wershil, S.J. Galli and M.H. Perdue. Substance P induces ion secretion in mouse small intestine through effects on enteric nerves and mast cells. *Am. J. Physiol.* 269: G85-92, 1995.
27. Yang, H., S. V. Wu, T. Ishikawa and Y. Taché. Cold exposure elevates thyrotropin-releasing hormone gene expression in medullary raphe nuclei: relationship with vagally mediated gastric erosions. *Neuroscience.* 61: 655-63, 1994.

The following work, my third publication, expands the original model concentrating on the increased epithelial permeability caused by acute stress being a pathophysiological response. We examined the impact of stress on the flux of protein across intestinal epithelium. We also examined which transport path was taken by the protein across the epithelium.

Chapter 4

Stress stimulates transepithelial macromolecular uptake in rat jejunum.

A.J. Kiliaan, P.R. Saunders, P.B. Bijlsma, C. Berin, J.A. Taminiou,
J.A. Groot and M.H. Perdue.

American Journal of Physiology, 1998. (submitted)

NOTE TO USERS

**Page(s) missing in number only; text follows.
Microfilmed as received.**

82

This reproduction is the best copy available.

UMI

ABSTRACT

Evidence suggests that stress may be a contributing factor in intestinal inflammatory disease; however, the involved mechanisms have not been elucidated. We previously reported that acute stress alters epithelial physiology of rat intestine. Here, we document stress-induced macromolecular transport across intestinal epithelium. Following exposure of Wistar Kyoto rats to acute restraint stress, transport of a model protein, horseradish peroxidase (HRP), was assessed in isolated segments of jejunum. The flux of intact HRP was significantly enhanced across intestine from stressed rats compared to controls. Electron microscopy revealed HRP-containing endosomes within enterocytes, goblet cells and Paneth cells of stressed rats. The number and area of HRP endosomes within enterocytes were found to be significantly increased by stress. HRP was also visualized in paracellular spaces between adjacent epithelial cells only in intestine from stressed rats. Atropine treatment of rats prevented the stress-induced abnormalities of protein transport. Our results suggest that stress, via mechanisms involving cholinergic pathways, causes epithelial dysfunction that includes enhanced uptake of macromolecular protein antigens. We speculate that immune reactions to such foreign proteins may initiate or exacerbate inflammation.

4.1 INTRODUCTION

For many years, stress and anxiety were thought to have an etiological role in chronic

inflammatory diseases and gastrointestinal pathophysiology. It was suggested that patients with inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) have an anxiety-prone personality profile [2,36]. Intestinal dysfunction was also correlated with depression [40]. Others argued that psychological problems occur as a result rather than a cause of chronic health problems [18]. A more recent report confirmed that stressful life events frequently precede disease relapses in patients with IBD [12]. However, the underlying mechanisms remain undefined.

Although numerous experimental studies have provided evidence that stress is involved in causing gastric ulceration [reviewed in 15] and altering intestinal motility [5,25,26,38], relatively few studies have examined the effects of stress on mucosal function [7,13]. The mucosa of the gastrointestinal tract consists of the lamina propria covered by a single cell layer of epithelial cells (mainly transporting enterocytes, but also goblet cells, enteroendocrine cells and Paneth cells) joined together by tight junctions that create a barrier restricting uptake of luminal material [22]. The lamina propria contains various immunocytes (mast cells, eosinophils, macrophages, neutrophils, lymphocytes, etc) and their numbers increase during inflammation. These cells react non-specifically to certain bacterial products or specifically to foreign protein antigens. In addition, the mucosa is highly innervated: networks of nerves surround the crypts, and nerve fibres extend into the villi with varicosities in close proximity to the epithelium [14]. Convincing evidence has been presented that enteric nerves regulate the transport function of the epithelium [8]. Signals can be communicated from the central nervous system to the gut via extrinsic nerves or their connections to intrinsic nerves in the plexus regions of the gut wall [39]. Although less is known about the nature of

extracellular signals regulating epithelial barrier function, it is reasonable to hypothesize that similar neural circuits might be involved.

We previously reported [32] that stress causes intestinal mucosal pathophysiology in Wistar Kyoto rats, a stress susceptible strain [27]. Following exposure of rats to restraint stress, jejunal preparations exhibited a secretory state, indicated by an increased baseline short-circuit current (I_{sc}) that was due to net Cl⁻ ion secretion. Stress (documented by elevated levels of corticosterone in blood samples obtained immediately following the protocol) also resulted in increased intestinal tissue conductance (G) and permeability to two small probes, mannitol and Cr-EDTA. These stress-induced changes occurred whether rats were restrained at either 22 °C or 8 °C; however, the magnitude of the intestinal response was greater at the lower temperature. A subsequent study [31] demonstrated that although both Wistar Kyoto rats and the parent Wistar strain responded to stress with intestinal epithelial transport abnormalities, the stress-induced changes were more profound in Wistar Kyoto rats, apparently due to a defect of intestinal cholinesterase activity resulting in hyper-responsiveness to cholinergic stimulation. In support of this hypothesis, atropine treatment of Wistar Kyoto rats prevented the transport abnormalities.

The current investigation was designed to determine if the stress-induced epithelial barrier defect, previously identified for small inert probes, extends to biologically relevant macromolecules such as protein antigens that might trigger an inflammatory/immune response. We examined protein uptake across isolated segments of intestine in Ussing chambers to eliminate any possible indirect effects of stress (e.g., changes in blood flow, motility or mucus secretion) that might affect protein transport *in vivo*. The study was

focused at the cellular level, on ultrastructural visualization of the transepithelial transport pathway. Under carefully controlled conditions, we found that stress stimulated protein transport via both the transcellular and paracellular pathways. Our studies implicated cholinergic mechanisms in enhanced uptake of macromolecules across the epithelium.

4.2 METHODS

4.2.1 Animals

The stress protocol was similar to that reported previously [31] and was approved by the Animal Care Committee at McMaster University. Adult male rats (mean weight 150 g) of the Wistar Kyoto strain were purchased from Charles River (St. Constant, Quebec, Canada). They were maintained on a normal light/dark cycle and provided with food and water ad libitum. Rats were handled daily by the same investigator for 2 weeks prior to study. Thirty min before the experiment, rats were injected ip with saline, atropine sulphate or atropine methyl nitrate (Sigma Chemical Co., St. Louis, MO; 10 mg/kg). This relatively high dose was based on our pilot studies and on reports that Wistar Kyoto rats have abnormally low levels of cholinesterase, both circulating and in gut mucosa [19,31]. In addition, we found that the Isc response to exogenous acetylcholine added to tissues in Ussing chambers was significantly greater in Wistar Kyoto rats than the parental Wistar strain [31], and that a greater concentration of atropine sulfate ($5 \times 10^{-5} \text{M}$) was required to inhibit the cholinergic component of the Isc response to transmural stimulation of enteric nerves in gut preparations from Wistar Kyoto rats [Saunders PR, unpublished observations]. Rats in the stress group were placed in a leucite adjustable restraining device (6 x 21.5 cm) for 2 h at 8°C, followed

by 2 h of free movement in their home cage at 22°C. We previously showed that epithelial ion transport abnormalities were maximal at 2 h post stress (although the levels of circulating corticosterone had normalized) and gradually returned to baseline values by 24 h [P. R. Saunders, unpublished observations; please see Appendix B]. Rats in the control group were also injected with atropine or saline, but remained in their home cage before study. Rats were then anaesthetized with im urethane and maintained at 37°C via a rectal probe and thermally controlled heating pad while a laparotomy was performed. A 15-20 cm segment of jejunum (beginning 5 cm distal to ligament of Treitz) was removed and placed in 37°C oxygenated Krebs buffer.

4.2.2 Ussing Chamber Studies

The jejunal segment was carefully stripped of external muscle and cut into 6-8 pieces that were returned to a large volume of 37°C oxygenated buffer. This rinsing removed any mediators that may have been released during the stripping process. The pieces were then mounted in Ussing chambers (opening of 0.6 cm²), taking care to avoid Peyer's patches. This entire procedure was completed within 5 min and tissues were always kept under physiological conditions of temperature, pH and oxygenation to avoid damage or deterioration that may influence macromolecular transport. With this experimental approach, tissues were viable for at least 3 h with consistent *G* measures and *Isc* responses to field stimulation. In the chambers, tissues were bathed in 37°C oxygenated Krebs buffer (10 ml on each side) containing (in mM): 115 NaCl, 1.25 CaCl₂, 1.2 MgCl₂, 2.0 K₂PO₄, 25 NaHCO₃, pH 7.35 ± 0.02. The serosal buffer included 10 mM glucose as an energy source, osmotically

balanced by 10 mM mannitol in the mucosal (luminal) buffer. The chambers contained agar-salt bridges to monitor the potential difference (PD) across the tissues and inject short-circuit current (Isc) to maintain zero PD as measured via an automatic voltage clamp (W-P Instruments, Narco Scientific, Downsview, Ont., Canada). Conductance (G) was calculated from Ohm's Law.

4.2.3 Permeability

The inert probe, $^{51}\text{Cr-EDTA}$ (6 $\mu\text{Ci/ml}$) (Radiopharmacy, Chedoke-McMaster Hospital, Hamilton, Canada), was added to the mucosal buffer of the Ussing chambers, balanced by an equivalent concentration of unlabelled Cr-EDTA in the serosal buffer. Samples, 1.0 ml from the serosal buffer and 0.05 ml from the mucosal buffer, were obtained at 30 min intervals or at the beginning and end of the experiment, respectively. Buffers were replaced as required to keep the volume constant. The radioactivity of $^{51}\text{Cr-EDTA}$ was measured in a gamma counter. Transepithelial fluxes were calculated by standard formulae and were expressed as $\text{nmol/cm}^2/\text{h}$.

4.2.4 Protein Transport

We used horseradish peroxidase (HRP) as a model protein since it has a molecular weight of 40 kD, similar in size to antigenic proteins known to stimulate immune responses in sensitive individuals [10]. Intact HRP can be quantitatively measured using an enzymatic assay and the reaction product is easily visualized as electron dense material in ultrastructural studies. HRP (10^{-5}M , type VI, Sigma) was added to the mucosal buffer. Samples, 0.5 ml from

the serosal buffer and 0.05 ml from the mucosal buffer, were obtained at 30 min intervals or at the beginning and end of the experiment, respectively. Buffers were replaced as required to keep the volume constant. A modified kinetic assay [4] was used to measure intact HRP. Briefly, the reaction mixture (0.8 ml) contained 0.003% H₂O₂ and 0.009% o-dianisidine di-HCl (Sigma) with 0.15 ml of sample added. The rate of appearance of reaction product was used to calculate flux values expressed as pmol/cm²/h.

4.2.5 Electron Microscopy

Tissues were removed at 60 and 120 min, fixed and processed for visualization of HRP reaction product by electron microscopy. Methods for HRP product identification were modified from Graham and Karnovsky [16]. The tissues were fixed in 2.5% glutaraldehyde in 0.1 M sodium-cacodylate buffer (pH 7.4) for 2 h at 22°C, rinsed for 18 h (4 °C) with 0.05 M TRIS buffer (pH 7.6) and then washed 3 times, 5 min each time. Peroxidase activity was demonstrated by incubating the tissues for 15 min in 0.5 mg/ml diaminobenzidine in 0.05 M TRIS buffer (pH 7.6, 22°C) and subsequently incubating for 15 min in the same buffer containing 0.01% H₂O₂. Tissues were then processed for routine electron microscopy. Quantitative analysis was performed on coded high magnification photomicrographs; 80 micrographs (i.e., at least 80 enterocytes) were evaluated per rat group (20 per rat in 4 rats per group). The number and diameter of HRP-containing endosomes were determined in 4 x 6 μm sized windows in the apical region (between the microvilli and the nucleus) of villus enterocytes, and the area occupied by such endosomes was calculated. Preliminary studies determined that endogenous peroxidase was not evident in epithelial cells or lamina propria

of tissues from control or stressed rats.

4.2.6 Statistical Analysis

ANOVA and subsequent Neuman-Keuls analyses were used to compare groups; single comparisons were performed using the Student's t-test. Pearson's coefficient was used to determine correlations. A value of $p < 0.05$ was accepted as statistically significant.

4.3 RESULTS

4.3.1 Tissue Conductance and Permeability to Cr-EDTA

Compared with controls, jejunal tissues obtained from stressed rats exhibited a significantly increased ($p < 0.05$) baseline conductance (Table 4.1). This abnormality was inhibited by treating rats with either atropine sulfate or atropine methyl nitrate before exposing them to stress. The stability of tissue conductance over time in all groups (i.e., lack of significant difference between initial values and those at the end of the experiment) indicated a lack of significant deterioration of the tissues during the 2 h experimental period. The flux of Cr-EDTA was also significantly increased ($p = 0.023$) across tissues from stressed rats compared with controls (Figure 4.1A). Again, treatment of rats with atropine sulfate abolished these stress-induced changes. There was a highly significant coefficient of correlation ($r = 0.88$, $p < 0.001$) for conductance values of individual tissues and their permeability to Cr-EDTA, as would be expected for paracellular transport of this small (MW 340) probe. Atropine sulfate did not affect intestinal conductance values or Cr-EDTA flux in control rats (Table 4.1, Figure 4.1A).

TABLE 4.1 Conductance values for jejunal tissues

Rat Group	20 min	120 min
Control	39.8 ± 1.9	38.7 ± 2.1
Control (atropine sulfate)	40.4 ± 2.9	43.3 ± 3.6
Stress	55.2 ± 5.5 *	49.6 ± 4.6*
Stress (atropine sulfate)	45.2 ± 2.8	42.0 ± 3.6
Stress (atropine methyl nitrate)	39.8 ± 0.9	43.4 ± 1.0

Conductance (expressed as mS/cm²) of jejunal tissues at the beginning (20 min) and end (120 min) of the experiment. Values shown represent the means ± SEM; 4 tissues were studied from each of 6-7 rats per group. Conductance was significantly increased ($p < 0.05$) in tissues from stressed rats compared with controls.

4.3.2 Protein Transport

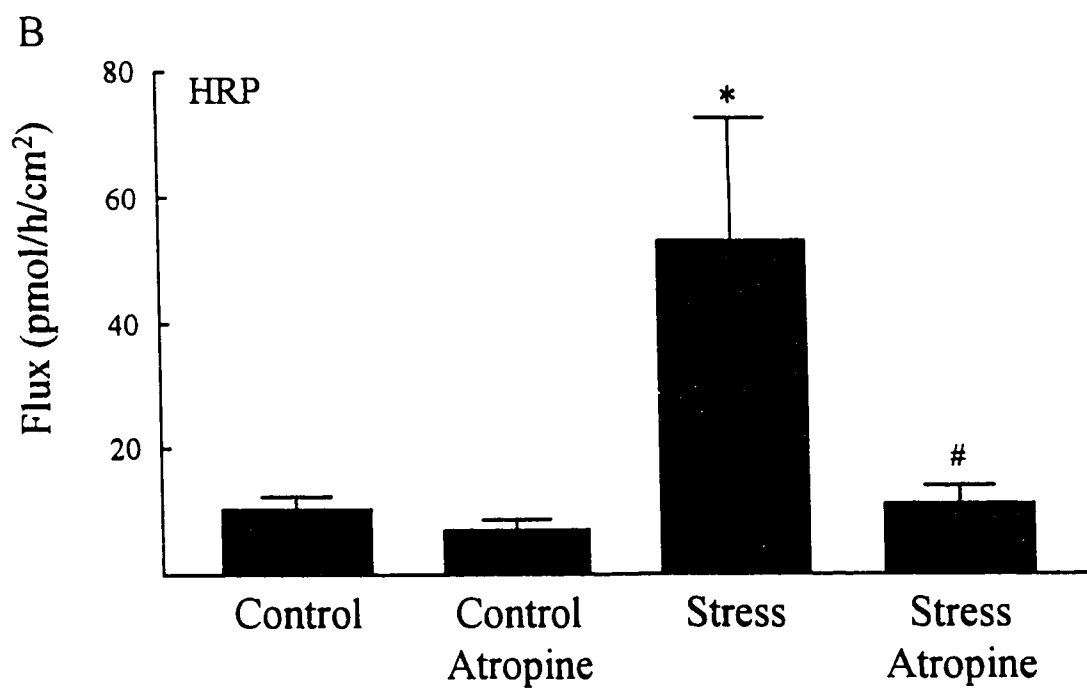
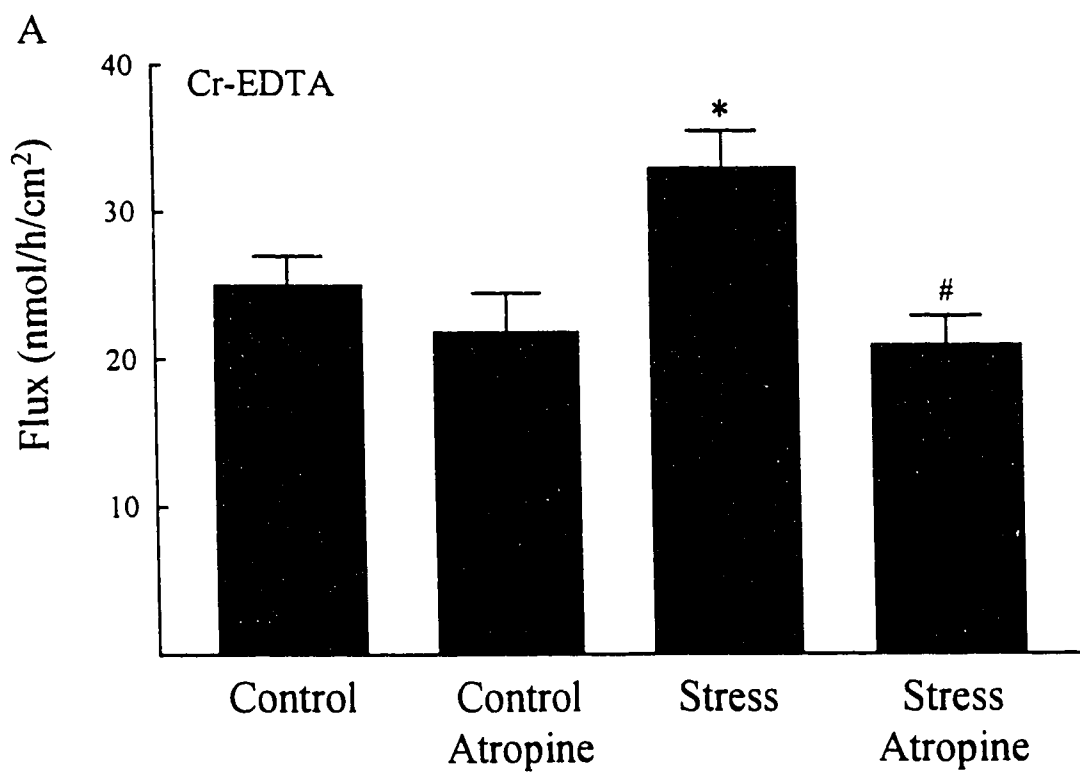
Figure 4.1B depicts the flux of HRP across tissues from stressed and control rats. Transport of HRP was dramatically enhanced by stress, with the flux value ~5 fold that across control tissues (53.1 ± 19.5 versus 10.3 ± 2.1 pmol/h/cm², mean ± SEM; $p = 0.026$). Figure 4.1B also shows that atropine sulfate prevented the increase in intestinal protein transport in stressed rats (11.1 ± 3.0 pmol/h/cm²), but had no effect in controls. Further experiments revealed a similar degree of inhibition with atropine methyl nitrate (14.6 ± 2.7 pmol/h/cm²; $n = 6$ rats).

4.3.3 Electron Microscopy

4.3.3.1 Transcellular Pathway

Eighty high power electron photomicrographs were evaluated per rat group to obtain qualitative and quantitative information on the uptake and pathway of HRP transport across

Figure 4.1 Effect of stress in atropine sulphate-treated or saline-treated rats on the intestinal transport of A. Cr-EDTA or B. horseradish peroxidase (HRP) following addition to the mucosal buffer of jejunal tissues in Ussing chambers. Bars indicate mucosal-to-serosal flux values expressed as means \pm SEM; 4 tissues were studied from each of 7 rats per group. The fluxes of Cr-EDTA and HRP were significantly increased across tissues from stressed rats compared with the other groups. Cr-EDTA: * $p = 0.023$, stress compared with control; # $p = 0.002$, stress/atropine compared with stress. HRP: * $p = 0.026$, stress compared with control, # $p = 0.029$, stress/atropine compared with stress.



the epithelium. No epithelial damage was observed in tissues from any rat group. HRP was clearly evident within endosomes in villus enterocytes of rats in all groups (Figure 4.2A-C). Endosomes appeared more dense and numerous in enterocytes from stressed rats (Figure 4.2B) compared with controls (Figure 4.2A) and atropine sulfate-treated stressed rats (Figure 4.2C). A lower power photomicrograph from a stressed rat (Figure 4.3C) shows a large number of HRP-containing endosomes located throughout enterocytes and goblet cells. In the crypts, HRP was observed in endosomes within enterocytes (not shown) and Paneth cells and also aggregated on the microvilli of these cells (Figure 4.2D).

The numbers of HRP-containing endosomes within $4 \times 6 \mu\text{m}$ windows in the apical region of villus enterocytes are shown in Figure 4.4A. A significantly greater ($p = 0.013$) number of HRP-positive endosomes were present in enterocytes of stressed rats (2.9 ± 0.3) compared with controls (1.4 ± 0.3). In addition, the area occupied by HRP was increased ~ 4 fold ($p = 0.015$) in stressed rats compared with controls (Figure 4.4B). Atropine sulfate treatment of rats prior to stress prevented the increased uptake of HRP. The amount of HRP in goblet cells was not quantified since the mucin content within the cells made this technically impossible, although our impression was that the pattern of transport was similar to that in enterocytes.

4.3.3.2 Paracellular Pathway

In tissues from control rats, HRP was not demonstrated in the paracellular spaces between adjacent epithelial cells (Figure 4.3A). In contrast, photomicrographs from 75% of stressed rats revealed HRP within paracellular spaces and tight junctions (Figure 4.3B). This finding was most evident at the 120 minute time point. Paracellular transport was apparent

both in the villus region where HRP penetrated the entire length of the lateral space (Figure 4.5A), and in the crypts (Figure 4.5B) where the depth of HRP penetration was incomplete but clearly past the tight junctional region in many instances. HRP was never observed in the paracellular spaces in stressed rats treated with atropine. HRP fluxes were higher in tissues from rats where paracellular HRP was demonstrated; a significant correlation ($r = 0.79$, $p = 0.002$) was found between conductance values of tissues from rats in all groups and HRP fluxes. These findings suggested that the paracellular route contributed to the higher protein transport induced by stress.

In the lamina propria, HRP was observed inside phagocytes, particularly eosinophils that appeared to be activated by this process since their granules were hypodense (Figure 4.5C). Some mast cells also appeared activated and released mast cell granules were found in the tissue (Figure 4.5D). Such observations were frequent in tissues from stressed rats but rare in tissues from control rats.

4.4 DISCUSSION

Two previous studies have reported that severe physical stress (surgical trauma or burns) perturbs the intestinal barrier [6,30]. To our knowledge, our study is the first to document that a short period of stress that was relatively mild (based on the normalization of corticosterone values at the time the rats were studied) enhances intestinal epithelial permeability to macromolecules. Overall transport of HRP was increased by stress and HRP was demonstrated to penetrate jejunal epithelium via both the transcellular and paracellular pathways. The number of HRP endosomes and their area inside enterocytes was significantly

Figure 4.2 Electron photomicrographs of intestinal tissues showing horseradish peroxidase-containing endosomes. **A.** Tissue from a control rat showing HRP product-containing endosomes (arrow) in the apical region of a villus enterocyte; MV indicates microvilli. **B.** Tissue from a stressed rat showing HRP product-containing endosomes (arrow) in the apical region of a villus enterocyte. **C.** Tissue from an atropine sulfate-treated stressed rat showing HRP product-containing endosomes (arrow) in the apical region of a villus enterocyte. **D.** Paneth cell in the crypt in tissue from a stressed rat showing HRP product adhering to the microvillus membrane and within endosomes (arrows). Bars = 1 μ m.

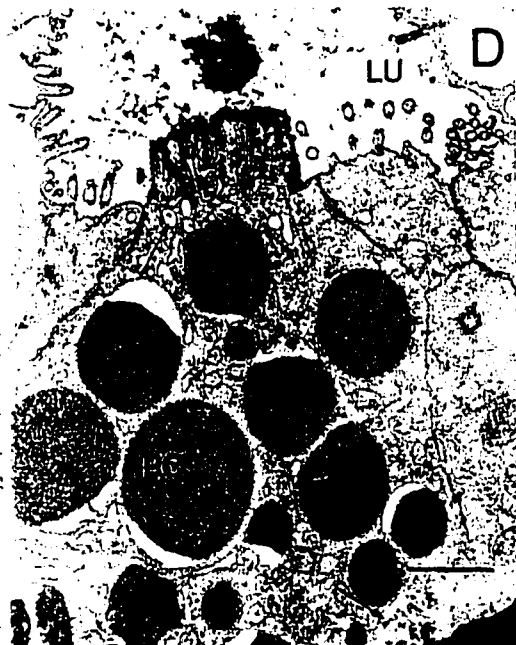


Figure 4.3 Electron photomicrographs of intestinal tissues showing horseradish peroxidase (HRP) in paracellular spaces between enterocytes and in endosomes within enterocytes. A Empty paracellular spaces and tight junctions (arrows) between 2 adjacent villus enterocytes in tissue from a control rat; bar = 0.5 μm . B. Horseradish peroxidase (HRP) product within paracellular spaces and tight junctions (arrows) in tissue from a stressed rat; bar = 0.5 μm . HRP was visualized frequently in paracellular spaces and tight junctions between villus enterocytes, and occasionally between crypt enterocytes, in tissues from stressed rats, but never in tissues from controls or atropine treated stressed rats. C. Lower power photomicrograph (bar = 3 μm) of villus epithelium in tissue from a stressed rat showing HRP-containing endosomes within enterocytes (arrows) and goblet cells (GC). The lumen (LU) is indicated at the top and the lamina propria at the bottom of the photomicrograph.

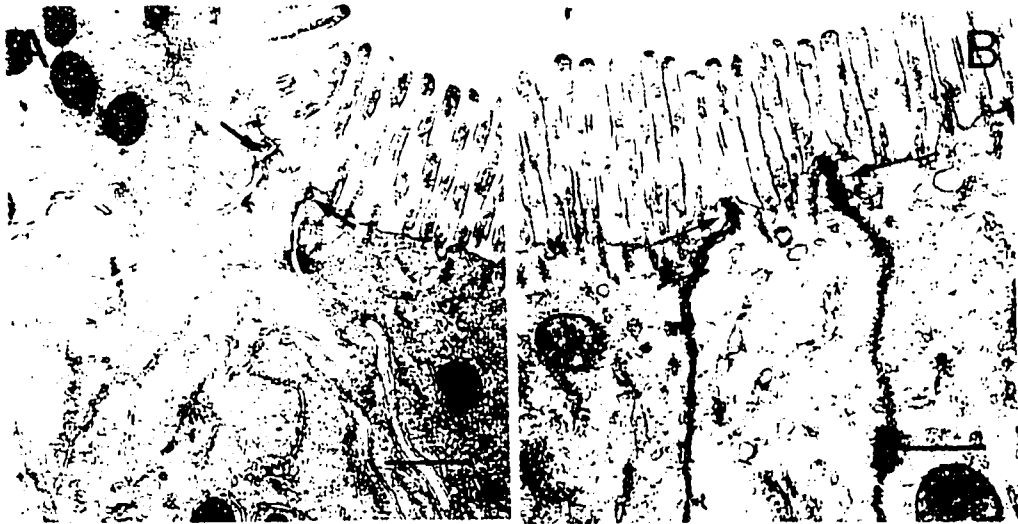


Figure 4.4 Effect of stress in atropine sulphate-treated or saline-treated rats on uptake of horseradish peroxidase into villus enterocytes. A. Number or B. area of HRP product-filled endosomes in enterocytes of tissues fixed 60 min after adding HRP to the mucosal buffer in Ussing chambers. Bars indicate values (expressed as means \pm SEM) for $4 \times 6 \mu\text{m}$ windows in the apical region (between the microvilli and the nucleus) of villus enterocytes; 20 such windows were evaluated for each rat, 4 rats per group (i.e., 80 enterocytes per rat); values per rat were averaged to obtain the values shown ($n = 4$). Endosome number and area were significantly increased in tissues from stress rats compared with the other groups. Number: * $p = 0.013$, stress compared with control; # $p = 0.002$, stress/atropine compared with stress; Area: * $p = 0.015$, stress compared with control; # $p = 0.005$, stress/atropine compared with stress.

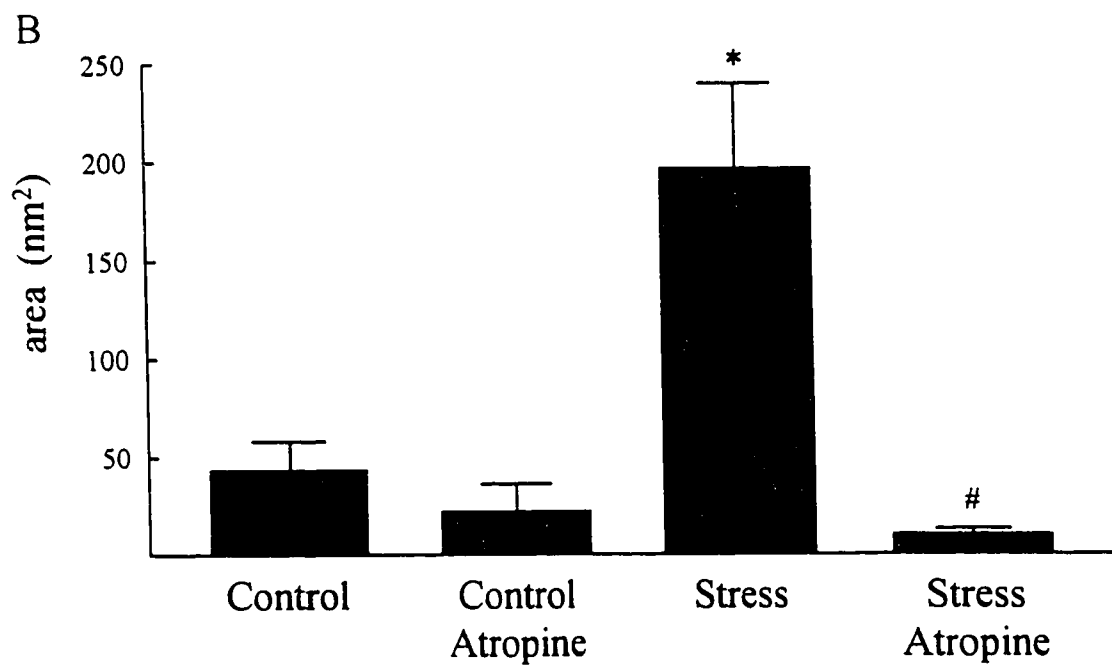
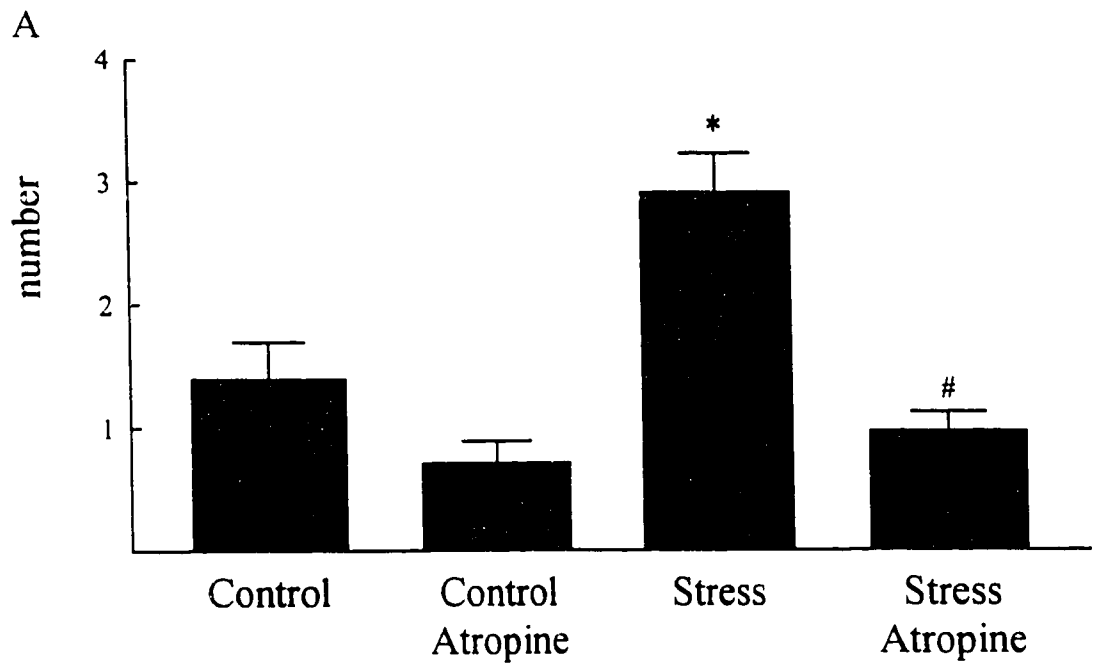
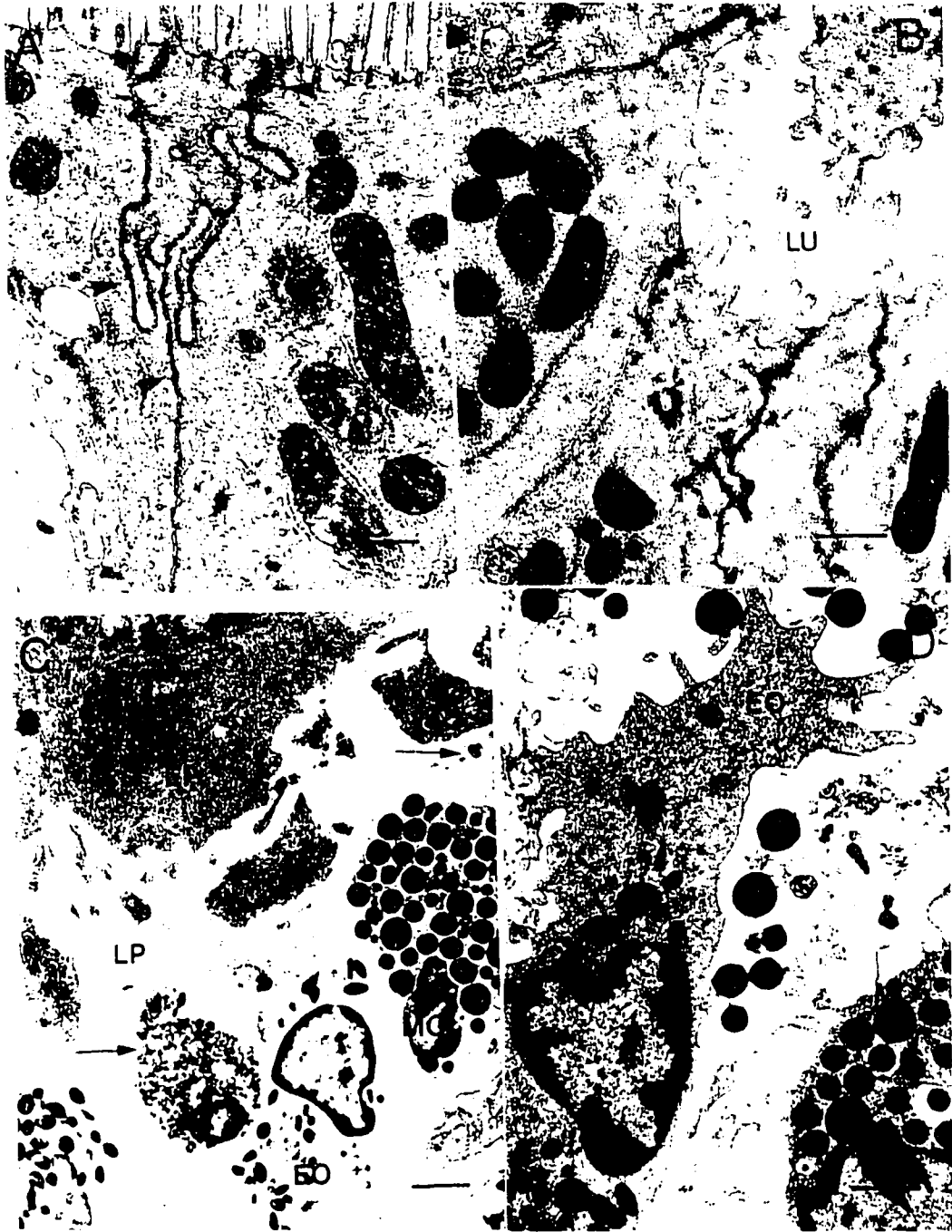


Figure 4.5 Electron photomicrographs of intestinal tissues showing horseradish peroxidase (HRP) in paracellular spaces between enterocytes and in the lamina propria beneath the epithelium. A. Horseradish peroxidase (HRP) product within paracellular spaces and tight junctions (arrows) between villus enterocytes in tissue from a stressed rat; bar = 0.5 μm . B. HRP entering paracellular spaces between crypt enterocytes in tissue from a stressed rat; bar = 0.5 μm . The lumen (LU) is on the right side of the photomicrograph. C. HRP (arrows) in the lamina propria (LP) beneath the epithelium (EP) and taken up by an eosinophil (EO) in tissue from a stressed rat; bar = 2 μm . A few hypodense granules are evident in the eosinophil suggesting activation. D. An eosinophil (EO) and mast cell (MC) in the lamina propria of tissue from a stressed rat; bar = 1 μm . Released mast cell granules are apparent between the cells.



increased by restraint stress. HRP-containing endosomes were also observed in goblet cells and Paneth cells of stressed rats. HRP was visualized in the paracellular spaces between adjacent epithelial cells in stressed but not in control rats. In addition, our study implicated cholinergic mechanisms in the stress-induced epithelial abnormalities since treatment of rats with muscarinic antagonists prevented the changes in barrier function.

Our earlier studies [32] documented that Wistar Kyoto rats react to acute stress with alterations in intestinal epithelial physiology. Ion secretion was stimulated and permeability to ions and the small inert probes, mannitol and Cr-EDTA, was enhanced. The transport abnormalities were maintained over the 3 h time of the experiment and did not return to normal values until 24 h later. We also recorded a reduced *I_{sc}* response to electric transmural stimulation of nerves in tissues from stressed rats. However, normal *I_{sc}* responses to several secretagogues were not affected by stress, suggesting that stress induced neurotransmitter release from intestinal mucosal neurons.

In a subsequent study [31], we determined that Wistar Kyoto rats respond to stress with epithelial abnormalities several fold greater than those in Wistar rats (the parent strain). Our finding that Wistar Kyoto rats have reduced activity of cholinesterase in intestinal mucosa suggested a role for cholinergic mechanisms in the gut abnormalities. Both atropine sulfate, a general muscarinic antagonist, and atropine methyl nitrate, a quaternary salt that does not cross the blood-brain barrier, inhibited the stress-induced intestinal pathophysiology. However, hexamethonium, a ganglionic blocker, had no effect. Those studies implicated peripheral cholinergic receptors in the stress-induced changes in intestinal mucosal function.

In this study, we demonstrated that stress resulted in an impairment of the epithelial

barrier to macromolecules and that cholinergic mechanisms were also involved in mediating this effect. Both atropine sulfate and atropine methyl nitrate treatment of rats prior to stress inhibited the increased conductance as well as the five-fold increase in HRP flux caused by acute restraint stress. Ultrastructural analysis showed that the number and size of endosomes in a fixed region of enterocytes were significantly increased after HRP addition to the mucosal surface of tissue from stressed rats. Again, muscarinic blockade prevented the stress-induced increases. These findings are compatible with those from previous studies that have implicated nerves in the regulation of epithelial barrier function. General and specific neural blockade have been demonstrated to inhibit transepithelial transport of proteins such as bovine serum albumin and ovalbumin [9,17]. Other studies [4,29] have shown that the cholinergic agonist, carbachol, increased transepithelial transport of HRP via transcellular and paracellular pathways in rat ileum. Carbachol also stimulated secretion of mucin from goblet cells [28], and stress has also been shown to induce mucus secretion [7]. Therefore, cholinergic stimulation may have been involved in the uptake of HRP into these cells. We found HRP bound to the apical membrane and within another secretory cell type in crypt epithelium, the Paneth cell. Our studies did not provide information on the possible mechanisms that might account for this effect.

Atropine sulfate treatment of rats prevented all of the epithelial permeability abnormalities we documented in response to stress, but had no effect on intestinal transport parameters in control rats. This suggests that the effects of atropine were specific on muscarinic receptors rather than due to non-specific central action. To obtain additional information on whether its effects were centrally mediated, we conducted experiments with

atropine methyl nitrate, a form that does not cross the blood-brain barrier. Atropine methyl nitrate produced nearly identical results to those with atropine sulfate: inhibition of stress-induced increases in conductance and macromolecular protein transport. Taken together, these results implicate peripheral rather than central sites for the muscarinic receptors involved in the regulation of macromolecular protein transport.

HRP within tight junctions and paracellular spaces was observed in photomicrographs of intestinal tissues obtained from stressed rats but never in photomicrographs of tissues from control rats or stressed rats treated with atropine sulfate. The protein was visible along the entire length of the intercellular spaces between villus cells, but penetration appeared to be more limited between crypt cells, possibly due to the reduced ability of the macromolecule to enter the crypts. The presence of HRP in the paracellular regions was more evident in sections fixed at later times, suggesting gradual accumulation of HRP in this pathway. Protein in the paracellular spaces of specific tissues was associated with increased conductance, and the conductance values also correlated with HRP fluxes. These findings suggest that stress enhances the permeability of the paracellular pathway, not only to Cr-EDTA, but also to macromolecular proteins.

Tight junctions are impermeable to proteins under normal circumstances [22], although physiological regulation of tight junctional permeability to ions and small molecules is recognized. However, under certain conditions, including cholinergic stimulation with carbachol, HRP penetration of the tight junctions has been reported [4]. Carbachol causes release of Ca^{++} from intracellular stores and activation of protein kinase C (PKC) in cultured epithelial cells [20]; activation of PKC has been implicated in loosening of tight junctions [35].

Decreased resistance of tight junctions in epithelial monolayers was shown to be associated with f-actin rearrangements and phosphorylation of myosin light chain in response to T cell activation or bacterial attachment [23,33]. Stress may be an extreme situation where cholinergic stimulation in combination with other factors may result in an increase in tight junctional permeability that extends to macromolecules.

Our studies implicate cholinergic nerves in the stressed-induced epithelial pathophysiology, but do not rule out other cells/mechanisms. Evidence indicates that mucosal immune cells, such as mast cells, may be in the pathway resulting in intestinal pathophysiology. Stress-induced colonic mucin secretion was shown to be mediated, at least in part, by mast cells [7]. Studies have reported that vagal stimulation can cause mediator release from mast cells in rat ileum [3]. In addition, acute stress resulted in release of substance P from guinea pig airways and chronic stress caused reduced tissue levels of substance P [1], a neuropeptide that can stimulate mast cells [33]. We previously demonstrated that intestinal mast cells could be activated via the nervous system by environmental cues following learned association by rats of these cues with antigen challenge [21]. The electron photomicrographs from this study showed eosinophils and other phagocytes in the lamina propria containing HRP. Reduced density and numbers of eosinophil granules and the presence of free mast cell granules in the lamina propria suggested, but did not prove, activation of these cells. However, it is clear that cholinergic nerves are critical in the pathway since atropine treatment of rats prevented the stress-induced epithelial barrier defect.

Our findings may be important for understanding the role of stress in allergic and

inflammatory conditions. Stress may increase epithelial permeability facilitating passage of protein antigens, including food antigens and microbial toxins/products, from the gut lumen. The excessive uptake of antigens may trigger an immune response. For example, stress in sensitive individuals may increase allergen uptake and result in a local anaphylactic reaction due to mast cell activation. Release of mast cell mediators, such as histamine, may also be enhanced by cholinergic mechanisms [37] and would further increase epithelial permeability [9] and stimulate secretory activity [11]. In addition, there is evidence that intracellular processing of antigens by enterocytes is necessary for T cell suppression of immune responses resulting in oral tolerance [23]. Epithelial penetration of proteins via the paracellular route would avoid such protective mechanisms. The consequence of transcellular passage of proteins through cells in the epithelium, such as goblet cells, remains to be determined. Our previous finding that the degree of epithelial dysfunction induced by stress varies in different rat strains together with results of this study, suggest that genetic factors relating to cholinergic sensitivity may be important in regulating the intestinal epithelial barrier and thus determining the predisposition of an individual to stress-induced intestinal inflammation.

The authors would like to acknowledge the expert technical assistance of Ms. M. Benjamin, Ms. G. Scholten and the staff of the Electron Microscopy Unit, Faculty of Health Sciences, McMaster University. This research was supported by a grant from the Medical Research Council of Canada.

4.5 REFERENCES

1. Abe T., S. Yoshihara, T. Ando, and T. Ichimura. The influence of frequency of cold-water bathing on the Substance P content of the airways in the guinea pigs. *Regul. Pept. Suppl.* 1: S29, 1992.
2. Almy T.P., F. Kern Jr., and M. Tulin. Alterations in colonic function in man under stress: experimental production of sigmoid spasm in healthy persons. *Gastroenterology* 12:425-436, 1949.
3. Bani-Sacchi T., M. Barattini, S. Bianchi, P. Blandina, S. Brunelleschi, R. Fantozzi, P.F. Mannaioni, and E. Masini. The release of histamine by parasympathetic stimulation in guinea-pig auricle and rat ileum. *J. Physiol.* 371:29-43, 1986.
4. Bijlsma P.B., A.J. Kiliaan, G. Scholten, M. Heyman, J.A. Groot, and J.A.J.M. Taminiou. Carbachol but not forskolin can increase mucosal to serosal transport of intact protein in rat ileum *in vitro*. *Am. J. Physiol.* 271: G147-G155, 1996.
5. Bueno L., S.M. Collins, and J.L. Junien. *Stress and Digestive Motility*. London; John Libby Publishers; 1989.
6. Carter E.A., R.G. Tompkins, E. Schiffrin, and J.F. Burke. Cutaneous thermal injury alters macromolecular permeability of rat small intestine. *Surgery* 107:335-341, 1990.
7. Castagliuolo I., J.T. LaMont, B. Qiu, S.M. Fleming, K.R. Bhaskar, S.T. Nikulasson, C. Kornetsky, and C. Pothoulakis. Acute stress causes mucin release from rat colon: role of corticotropin releasing factor and mast cells. *Am. J. Physiol.* 271: G884-G892, 1996.
8. Cooke H.J. and R.A. Reddix. Neural regulation of intestinal electrolyte transport. In: Johnson LR, ed. *Physiology of the Gastrointestinal Tract*. Volume 2. 3rd ed.; New York; Raven Press; 1994: 2083-2132.
9. Crowe S.E., K. Soda, A.M. Stanisz, and M.H. Perdue. Intestinal permeability in allergic rats: nerve involvement in antigen induced changes. *Am. J. Physiol.* 264: G617-G623, 1993.
10. Crowe S.E. and M.H. Perdue. Gastrointestinal food hypersensitivity: basic mechanisms of pathophysiology. *Gastroenterology* 103:1075-1095, 1992.

11. Crowe S.E., P. Sestini and M.H. Perdue. Allergic reactions of rat jejunal mucosa. Ion transport response to luminal antigen and inflammatory mediators. *Gastroenterology* 99: 74-82, 1990.
12. Duffy L.C., M.A. Zielesny, J.R. Marshall, T.E. Byers, M.M. Weiser, J.F. Phillips, B.M. Calkins, P.L. Ogra and S. Graham. Relevance of major stress events as an indicator of disease activity prevalence in inflammatory bowel disease. *Behav. Med.* 17:101-110, 1991.
13. Empey, L.R. and R.N. Fedorak. Effect of misoprostol in preventing stress-induced intestinal fluid secretion in rats. *Prostaglandins Leukotrienes Essent. Fatty Acids.* 38: 43-8, 1989.
14. Furness J.B. and M. Costa. *The Enteric Nervous System*. New York; Churchill Livingstone; 1987.
15. Glavin G.B. Restraint ulcer: history, current research and future implications. *Brain Res. Bull.* 5:51-58, 1980.
16. Graham R.C. and M.J. Karnovsky. Glomerular permeability: ultrastructural cytochemical studies using peroxidases as protein tracers. *J. Exp. Med.* 124:1123-1134, 1966.
17. Kimm, M.H., G.H. Curtis, J.A. Hardin, and D.G. Gall. Transport of bovine serum albumin across rat jejunum: role of the enteric nervous system. *Am. J. Physiol.* 266: G186-G193, 1994.
18. Kirsner J.B. Ulcerative colitis 1970 - recent developments. *Scand. J. Gastroenterol.* 6 (suppl 6):63-91, 1970.
19. Lim, D.K., I.B. Hoskins, R.W. Rockhold and I.K. Ho. Comparative studies of muscarinic and dopamine receptors in three strains of rat. *Eur. J. Pharmacol.* 165: 279-87, 1989.
20. Luo H., R.P. Lindeman and H.S. Chase Jr.. Participation of protein kinase C in desensitization to bradykinin and to carbachol in MDCK cells. *Am. J. Physiol.* 262: F499-F506, 1992.
21. MacQueen G, Marshall J, Perdue M, Siegel S, Bienenstock J. Pavlovian conditioning of rat mucosal mast cells to secrete rat mast cell protease II. *Science.* 243:83-85, 1989.

22. Madara J.L. Loosening tight junctions - lessons from the intestine. *J. Clin. Invest.* 83: 1089-1094, 1989.
23. Mayer L. And D. Eisenhardt. Lack of induction of suppressor T cells by intestinal epithelial cells from patients with inflammatory bowel disease. *J. Clin. Invest.* 86: 1255-1260, 1990.
24. McKay D.M., K. Croitoru and M.H. Perdue. T cell-monocyte interactions regulate epithelial physiology in a coculture model of inflammation. *Am. J. Physiol.* 270: C418-C428, 1996.
25. McRae S., K. Younger, D.G. Thompson and D.L. Wingate. Sustained mental stress alters human jejunal motor activity. *Gut* 23:404-409, 1982.
26. Monnikes, H., B.G. Schmidt, H.E. Raybould and Y. Taché. CRF in the paraventricular nucleus mediates gastric and colonic motor response to restraint stress. *Am. J. Physiol.* 262: G137-43, 1992.
27. Pare W.P. Stress ulcer susceptibility and depression in Wistar Kyoto (WKY) rats. *Physiol. Behav.* 46:993-998, 1989.
28. Phillips T.E. Both crypt and villus intestinal goblet cells secrete mucin in response to cholinergic stimulation. *Am. J. Physiol.* 262: G327-G331, 1992.
29. Phillips T.E., T.L. Phillips and M.R. Neutra. Macromolecules can pass through occluding junctions of rat ileal epithelium during cholinergic stimulation. *Cell Tissue Res.* 247:547-554, 1987.
30. Rhodes R.S. and M.J. Karnovsky. Loss of macromolecular barrier function associated with surgical trauma to the intestine. *Lab. Invest.* 25:220-229, 1971.
31. Saunders P.R., N.P.M. Hanssen, D.S. Chandraratne and M.H. Perdue. Cholinergic nerves mediate stress-induced intestinal transport abnormalities in Wistar Kyoto rats. *Am. J. Physiol.* 273: G486-G490, 1997.
32. Saunders P.R., U. Kosecka, D.M. McKay and M.H. Perdue. Acute stressors stimulate ion secretion and increase epithelial permeability in rat intestine. *Am. J. Physiol.* 267: G794-G799, 1994.
33. Shanahan F, J.A. Denburg, J. Fox, J. Bienenstock and A.D. Befus. Mast cell heterogeneity: effects of the neuroenteric peptides on histamine release. *J. Immunol.* 135: 1331-1337, 1985.

34. Spitz J., R. Yuhan, A. Koutsouris, C. Blatt, J. Alverdy and G. Hecht. Enteropathogenic *Escherichia coli* adherence to intestinal epithelial monolayers diminishes barrier function. *Am. J. Physiol.* 268: G374-G379, 1995.
35. Stenson W.F., R. A. Easom, T.E. Riehl and J. Turk. Regulation of paracellular permeability in Caco-2 cell monolayers by protein kinase C. *Am. J. Physiol.* 265: G955-G962, 1993.
36. Sullivan A. Psychogenic factors in ulcerative colitis. *Am. J. Dig. Dis.* 2:651-656, 1935.
37. Wang L., A.M. Stanisz, B.K. Wershil, S.J. Galli and M.H. Perdue. Substance P induces ion secretion in mouse small intestine through effects on enteric nerves and mast cells. *Am. J. Physiol.* 269: G85-G92, 1995.
38. Wittman T., F. Crenner, F. Angel, L. Hanusz, C. Ringwald and J.F. Grenier. Long duration stress. Immediate and late effects on small and large bowel motility in rat. *Dig. Dis. Sci.* 35: 495-500, 1990.
39. Wood, J.D. Enteric neurophysiology. *Am. J. Physiol.* 247: G585-G598, 1984.
40. Young S.J., D.H. Alpers, C.C. Norland and R.A. Woodruff. Psychiatric illness and the irritable bowel syndrome. Practical implications for the primary physician. *Gastroenterology* 70:162-166, 1976.

NOTE TO USERS

**Page(s) missing in number only; text follows.
Microfilmed as received.**

113

This reproduction is the best copy available.

UMI

The following work, my fourth publication, addresses the effects of acute stress on intestinal (colonic) epithelial physiology. All the previous work has examined the effects of stress on jejunal epithelial physiology. To justify using this model to explore how stress may help mediate relapses in intestinal disease, the effects of stress must be seen in intestinal sites other than just the jejunum. We incorporated the most significant findings from the three previous papers to examine the impact acute stress has on the colonic epithelium.

Chapter 5

Stress-induced epithelial pathophysiology in rat colon: role of corticotropin-releasing factor.

P.R. Saunders, N.P.M. Hanssen, P-C. Yang, D. Yates, J.A. Groot and M.H. Perdue.

(in preparation)

NOTE TO USERS

**Page(s) missing in number only; text follows.
Microfilmed as received.**

116

This reproduction is the best copy available.

UMI

ABSTRACT

Evidence suggests that stress may be a contributing factor in intestinal inflammatory disease; however, the involved mechanisms have not been elucidated. We have previously reported that acute stress alters epithelial physiology of rat jejunum. In this study, we examined stress-induced functional changes in colonic mucosa from both Wistar and Wistar Kyoto rats. Only the Wistar Kyoto rats altered epithelial permeability in response to stress. Following exposure of these rats to acute restraint stress, the colonic epithelium demonstrated significantly increased baseline conductance of the tissue, increased flux of both the small bacterial tripeptide fMLP, and the large protein, horseradish peroxidase (HRP). Electron microscopy revealed an elevated content of HRP-containing endosomes within colonocytes. Pretreating rats with α helical-CRF₉₋₄₁ partially corrected the stress-induced abnormalities observed in the colon, while administration of exogenous corticotropin-releasing factor (CRF) mimicked the stress responses in the colon. Our results suggest that stress, via pathways that involve peripheral CRF, causes the epithelial barrier of the colon to be compromised in Wistar Kyoto rats. We speculate that stress susceptible individuals can develop a leaky epithelium allowing more noxious material/foreign antigens to enter the mucosa and initiate or exacerbate relapses of inflammation.

5.1 INTRODUCTION

Stress and anxiety are thought to contribute to symptoms in chronic inflammatory diseases and functional disorders of the gastrointestinal tract. Patients with inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) tend to have an anxiety-prone personality profile [1,20] and intestinal dysfunction has been correlated with depression [24]. A more recent report confirmed that stressful life events frequently precede disease relapses in patients with IBD [8]. However, the mechanism underlying the involvement of stress in intestinal pathophysiology remain undefined.

Although numerous experimental studies have provided evidence that stress causes gastric ulceration [reviewed in 10] and increases colonic motor activity [5,16,23], relatively few studies have examined the effects of stress on colonic mucosal function. One study reported that subjecting rats to cold-restraint stress reversed the typical colonic water absorption to secretion [9]. Another study showed that wrap restraint caused a dramatic increase in rat colonic mucous production [6]. While activity-stress (wheel running) over a period of a few days caused an overall thinning of the rat colon which was predominantly due to mucosal shrinkage (blunted crypts and depleted goblet cells); there was also evidence of hyperaemia and increased numbers of small polymorphonuclear leukocytes [2].

We previously reported [19] that stress causes mucosal pathophysiology in jejunum from Wistar Kyoto rats, a stress susceptible strain [17]. Following exposure of rats to restraint stress, with or without cold (8°C), jejunal preparations exhibited a secretory state, indicated by an increased baseline short-circuit current (I_{sc}) that was due to net Cl⁻ ion secretion. Stress also resulted in increased conductance and permeability to two small probes,

mannitol and Cr-EDTA. Reduced temperature enhanced the magnitude of the intestinal responses to stress but did not alter the responses qualitatively. A subsequent study [18] demonstrated that although both Wistar Kyoto rats and the parent Wistar strain responded to stress with intestinal epithelial transport abnormalities, the stress-induced changes were more profound in Wistar Kyoto rats, partially due to a defect of intestinal cholinesterase activity resulting in hyper-responsiveness to cholinergic stimulation. In confirmation of this hypothesis, atropine treatment of Wistar Kyoto rats prevented the transport abnormalities. Our last study [13] expanded the earlier findings to show that stress increased macromolecular permeability across jejunal tissues. The flux of the model protein, horseradish peroxidase (HRP), was increased in tissues from stressed Wistar Kyoto rats compared to control tissues. Electron microscopic analysis revealed that stress increased intracellular uptake of HRP and also induced paracellular transport of the protein. Atropine treatment prevented the stress-induced increase in macromolecular transport across the jejunal epithelium.

The current investigation was designed to explore if similar stress-induced epithelial pathophysiology occurs in the colon, a site with a higher prevalence for inflammatory bowel disease (IBD). Distal colonic segments from Wistar Kyoto rats subjected to acute stress compared with controls demonstrated both ion transport and permeability abnormalities; however, we concentrated on the barrier defect since it may play an important role in the disease process. We documented that restraint stress resulted in increased conductance of colonic tissues. In addition, fluxes across colon segments were significantly enhanced for the bacterial tripeptide, N-formyl-methionyl-leucyl-phenylalanine (fMLP), and the macromolecule

HRP. Electron microscopic analysis revealed increased uptake of HRP into colonic epithelial cells in stressed rats. We compared the findings observed in the Wistar Kyoto rats to the stress effects seen in distal colonic mucosa from the parent Wistar strain. Stress caused a greater ion transport perturbation in Wistars than Wistar Kyoto rats, however, stress did not induce any dramatic permeability changes in the Wistar rats. In addition, we show that peripheral corticotropin-releasing factor (CRF) is also a mediator of the stress-induced pathophysiology in the distal colon of Wistar Kyoto rats.

5.2 METHODS

5.2.1 Animal Model

Adult male rats (weight at time of study, 260-325 g) of the Wistar Kyoto and Wistar strain were purchased from Charles River (St. Constant, Quebec, Canada). They were maintained on a normal light/dark cycle and provided with food and water ad libitum. Rats were handled daily by the same investigator for 2 weeks prior to study to prevent any uncontrolled effects of stress. Rats in the stress group were placed in a leucite adjustable restraining device (6 x 21.5 cm) for 2 h at 4°C, followed by 2 h of free movement in their home cage at 22°C. We previously showed that epithelial ion transport abnormalities in the jejunum were maximal at 2 h post stress and gradually returned to normal by 24 h (please see Appendix B). Control rats were maintained in their home cage. Rats were then anaesthetized with urethane and maintained for 10-15 min on a heating pad at 37°C. The rats were laparotomized and the colon removed and placed in 37°C oxygenated Krebs buffer. All the experiments were approved by the Animal Care Committee at McMaster University.

5.2.2 Drug Administration

5.2.2.1 Atropine

To determine the role of cholinergic nerves in the stress-induced intestinal changes, rats were injected, ip, with either saline or 10 mg/kg atropine sulphate (Sigma Chemical Co., St. Louis, MO) 30 min prior to the stress protocol. This relatively high dose was required since Wistar Kyoto rats have abnormally low circulating and intestinal mucosal cholinesterase activity resulting in these rats being hypercholinergic [14,18].

5.2.2.2 CRF/CRF Antagonist

To determine if endogenous peripheral CRF contributed to the colonic stress responses, rats were injected, ip, with either saline or 50 µg/rat α helical-CRF₉₋₄₁ (Pennisula Laboratories, Belmont, CA.) 30 min prior to the stress protocol. Finally, control rats were injected, ip, with 10 µg/rat CRF (Pennisula Laboratories) 4 h before study to determine if CRF would induce similar abnormalities of colonic function.

5.2.3 Ussing chamber studies

The distal half of the colon was removed, stripped of external muscle and placed in warmed oxygenated Krebs buffer. From this segment 4 adjacent pieces were mounted in Ussing chambers (opening 0.6 cm²). Tissues were bathed in 37°C oxygenated Krebs buffer (8-10 ml on each side) containing (in mM): 115 NaCl, 1.25 CaCl₂, 1.2 MgCl₂, 2.0 K₂PO₄, 25 NaHCO₃, pH 7.35 ± 0.02. The serosal buffer included 10 mM glucose as an energy source, osmotically balanced by 10 mM mannitol in the mucosal (luminal) buffer. The chambers contained agar-salt bridges to monitor the potential difference (PD) across the tissues and

introduce a short-circuit current (I_{sc}) to maintain zero PD as measured via an automatic voltage clamp (W-P Instruments, Narco Scientific, Downsview, Ont., Canada). Conductance (G) was calculated from Ohm's law. tissues were viable for at least 3 h with consistent conductance measures and I_{sc} responses to field stimulation.

5.2.4 Permeability

The radiolabelled bacterial tripeptide, ^3H -fMLP (DuPont; Willmington, De.), was added to the mucosal buffer (1 $\mu\text{Ci/ml}$) and brought up to 10^{-5}M by cold fMLP (Sigma). Following a 25 min equilibration period 1.0 ml samples of serosal buffer were taken every 20 min and 0.05 ml samples of mucosal buffer were taken at the beginning and end of the experiment to determine the specific activity. Buffers were replaced as required to keep the volume constant. The radioactivity of ^3H -fMLP was measured in a beta counter. Transepithelial fluxes were calculated by standard formulae and were expressed as $\mu\text{mol/h/cm}^2$.

5.2.5 Protein transport

We used HRP as a model macromolecular protein similar in size (40kD) to antigenic proteins known to stimulate immune responses in sensitive individuals [7]. Intact HRP can be quantitatively measured using an enzymatic assay and the reaction product visualized as electron dense material in ultrastructural studies. We added 10^{-5}M of type VI HRP (Sigma) to the mucosal buffer. Following a 60 min equilibration period 0.5 ml samples of serosal buffer were taken every 30 min and 0.01 ml samples of mucosal buffer were taken at the

beginning and end of the experiment to determine the specific activity. Buffers were replaced as required to keep the volume constant. A modified kinetic assay [4] was used to measure HRP activity. Briefly, the phosphate buffer reaction mixture (0.8 ml, pH 6.0) contained 0.003% H₂O₂ and 0.009% o-dianisidine di-HCl (Sigma) and 0.15 ml of sample was added and read immediately in a spectrophotometer (λ 460 nm). The rate of appearance of reaction product was used as an indicator of the quantity of intact HRP in the sample and to calculate the transepithelial fluxes which were expressed as pmol/h/cm².

5.2.6 Electron microscopy

Tissues were removed at 120 min after the addition of HRP into the chambers, fixed and processed for visualization of HRP reaction product by electron microscopy. Methods for HRP product identification were modified from Graham and Karnovsky [11]. The tissues were fixed in 2.5% glutaraldehyde in 0.1 M sodium-cacodylate buffer (pH 7.4) for 2 h at 22°C, rinsed for 18 h (4°C) with 0.05 TRIS buffer (pH 7.6) and then washed 3 times, 5 min each time. Peroxidase activity was demonstrated by incubating the tissues for 15 min in 0.5 mg/ml diaminobenzidine in 0.05 M TRIS buffer (pH 7.6, 22°C) and subsequently incubating for 15 min in the same buffer containing 0.01% H₂O₂. Tissues were then processed for routine transmission electron microscopy. Quantitative analysis was performed on coded high magnification photomicrographs; 60 micrographs per group were evaluated (with 3-4 tissues from each of 4 rats/group). The area of HRP positive endosomes within colonic enterocytes was calculated for the apical (between the microvilli and the nucleus), the mid (adjacent to the nucleus), and the basal regions (between the nucleus and the basal membrane) and

reported as $\mu\text{m}^2/\text{window}$ (window area $25 \times 30 \mu\text{m}^2$). All tissues were examined for any evidence of paracellular HRP transport.

5.2.7 Statistical Analysis

ANOVA and subsequent Neuman-Keuls analyses were used to compare groups; single comparisons were performed using the Student t-test. A value of $p < 0.05$ was accepted as statistically significant.

5.3 RESULTS

5.3.1 Intestinal Parameters in Control and Stressed Wistar and Wistar Kyoto Rats

Table 5.1 shows that the baseline I_{sc} of colonic mucosa was significantly elevated following stress in both Wistar ($\Delta I_{sc} 48.8 \mu\text{A}/\text{cm}^2$) and Wistar Kyoto rats ($\Delta I_{sc} 19.1 \mu\text{A}/\text{cm}^2$). The baseline I_{sc} of control tissue from the Wistar Kyoto strain is much greater compared to controls from the Wistar strain. However, a significant increase in baseline conductance, following stress, was observed only in tissue from Wistar Kyoto rats ($\Delta G 10.8 \text{ mS}/\text{cm}^2$ vs. $4.7 \text{ mS}/\text{cm}^2$ for Wistars). In addition, significant increases in epithelial molecular permeability to both small and large molecules was observed only in the Wistar Kyoto strain. Figure 5.1A shows fluxes of the bacterial tri-peptide fMLP, indicating that tissue from stressed Wistar Kyoto rats is twice as permeable (201%) for this probe compared to control tissue, while the tissue from Wistar rats is one and a half times as permeable (154%) compared to controls. Figure 5.1B shows fluxes for the macromolecular probe HRP, indicating that tissue from stressed Wistar Kyoto rats is more than twice as permeable (259%) for this probe compared

to control tissue, while the tissue from Wistar rats is a little more than one and a half times as permeable (170%) compared to controls. Please note that even though tissue from stressed Wistar rats had, on average, higher fluxes for fMLP and HRP, these fluxes were not significantly different from the control fluxes.

TABLE 5.1 Electrophysiological Parameters in Colonic Mucosa from Wistar and Wistar Kyoto Rats

	I_{sc} ($\mu\text{A}/\text{cm}^2$)		G (mS/cm^2)	
	control	stress	control	stress
Wistar	36.3 ± 6.2	$85.1 \pm 11.4 *$	15.0 ± 1.8	19.7 ± 2.2
Wistar Kyoto	68.2 ± 7.3	$87.3 \pm 8.0 *$	15.0 ± 1.0	$25.8 \pm 1.7 *$

Baseline short-circuit current (I_{sc}) and conductance (G) were recorded at equilibrium (20 min after mounting). There were significant differences from control for the baseline I_{sc} measures in both the Wistar ($p=0.002$) and Wistar Kyoto ($p=0.036$) strains. A significant difference from control for baseline conductance readings was only evident in the Wistar Kyoto strain ($p<0.001$). Values represent means \pm SEM; $n = 10-14$ rats per group with 3-4 tissues averaged to give a value per rat. * indicates group significantly different from control.

5.3.2 Electron microscopy

Further studies were carried out to examine the stress-induced epithelial barrier defects in Wistar Kyoto rats. High power electron photomicrographs were evaluated from stressed and control rats to obtain qualitative and quantitative information on the uptake and pathway of HRP transport across the epithelium. HRP was clearly evident within endosomes in enterocytes of rats in both groups (Figure 5.2A-B). Endosomes appeared more dense and numerous in enterocytes from stressed rats compared with controls. The area of HRP-containing endosomes in the apical, mid and basal regions of colonic enterocytes are shown in figure 5.3. The percentage of control values are 366% for the apical region, 283% for the

Figure 5.1 Effect of stress on colonic transport in Wistar and Wistar Kyoto rats. Fluxes of **A.** the bacterial tripeptide formyl-met-leu-phe (fMLP) or **B.** horseradish peroxidase (HRP) from stripped colonic tissues in Ussing chambers. The mucosal-to-serosal fluxes of fMLP and HRP were significantly increased across tissues from stressed rats (solid bars) compared with controls (open bars) for only the Wistar Kyoto rats; fMLP * $p = 0.045$, HRP * $p = 0.029$. Values are means \pm SEM for 2-4 tissues averaged per rat for 5-6 rats per group.

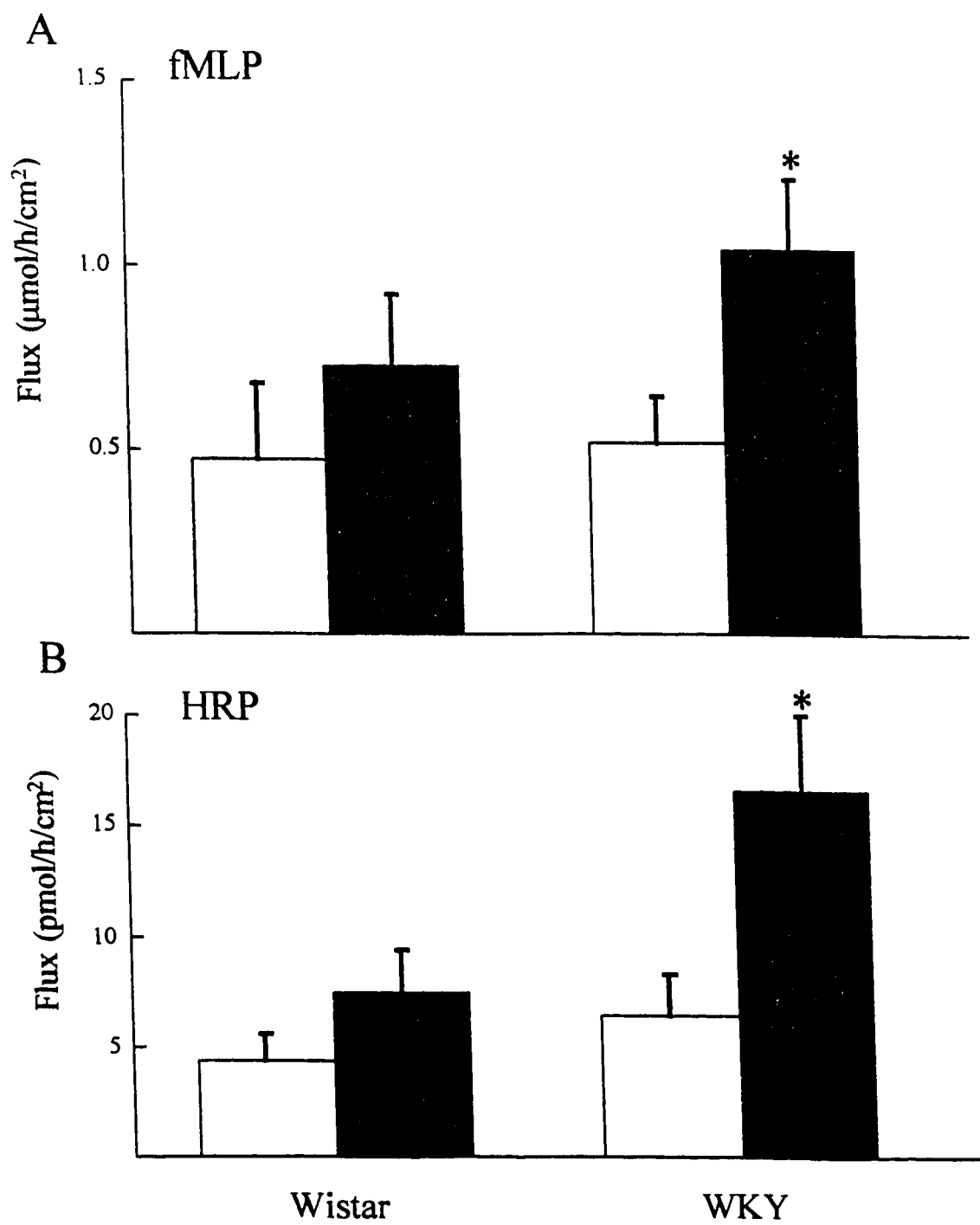
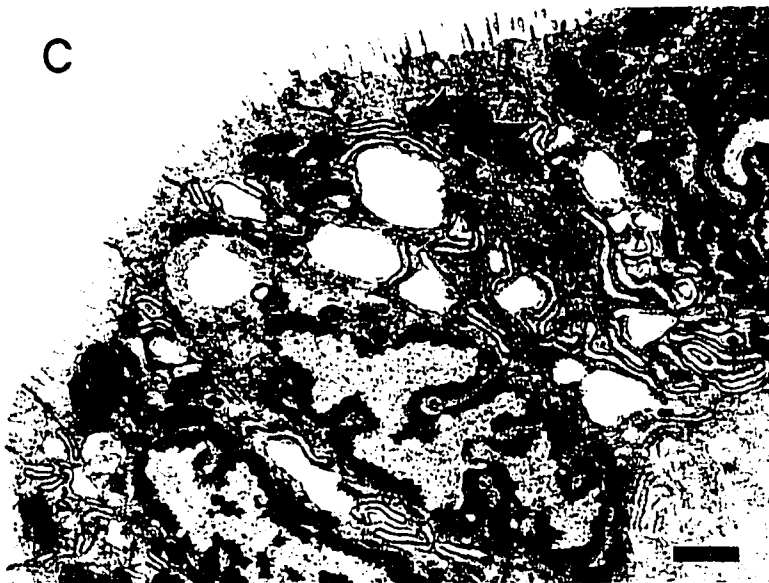
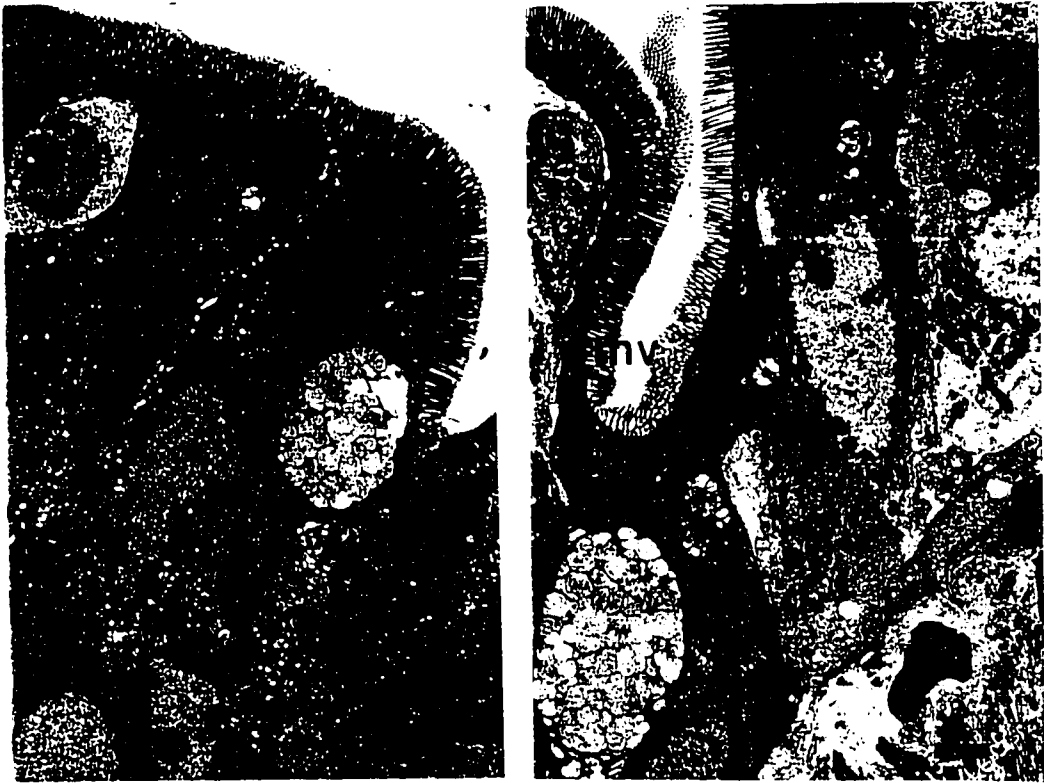


Figure 5.2 Electron photomicrographs of intestinal tissues showing horseradish peroxidase-containing endosomes. **A.** Tissue from a control rat, and **B.** tissue from a stressed rat, 120 minutes after incubation of 10^{-5} M HRP on the luminal side. HRP product-containing endosomes (arrows) are seen in the colonic enterocytes. **MV** indicates microvilli. **C.** The paracellular spaces between colonic enterocytes, of tissue from stressed rats 120 minutes after the addition of HRP, contain HRP beyond the level of the tight junctions (arrows). Scale bars are 2 μ m for **A** & **B** and 1 μ m for **C**.



mid region, and 192% for the basal region of the enterocytes. A significantly greater total area of HRP-positive endosomes were present in all three regions of enterocytes in stressed rats compared with controls. On occasion the reaction product to HRP could be observed in the paracellular spaces right up to the level of the tight junctions (Figure 5.2C).

5.3.3 Mediators of the Stress Response

5.3.3.1 Acetylcholine

We had previously shown that pretreating Wistar Kyoto rats with atropine prevented the stress induced epithelial pathophysiology observed in the jejunum [18]. To determine if cholinergic factors play a similar critical role in the stress-induced gut pathophysiology in the colon, Wistar Kyoto rats were treated with atropine sulphate prior to the stress protocol. Atropine treatment of control rats had no effect on I_{sc} or conductance. There was the trend that atropine corrected part of the stress-induced pathophysiology in the colon. The increased baseline I_{sc} was diminished by 38.4%, the increased baseline conductance by 32.9%, and the increased flux of HRP by 38.2% (Table 5.2). These measures were not significantly different from the stress group, however, these measures were also not significantly different from control measures.

5.3.3.2 CRF

Pretreating Wistar Kyoto rats with the CRF antagonist, α helical-CRF₉₋₄₁, prior to stress partially corrected the stress-induced pathophysiology observed in the colonic mucosa. CRF antagonism prevented the stress-induced increase in baseline I_{sc} diminishing it

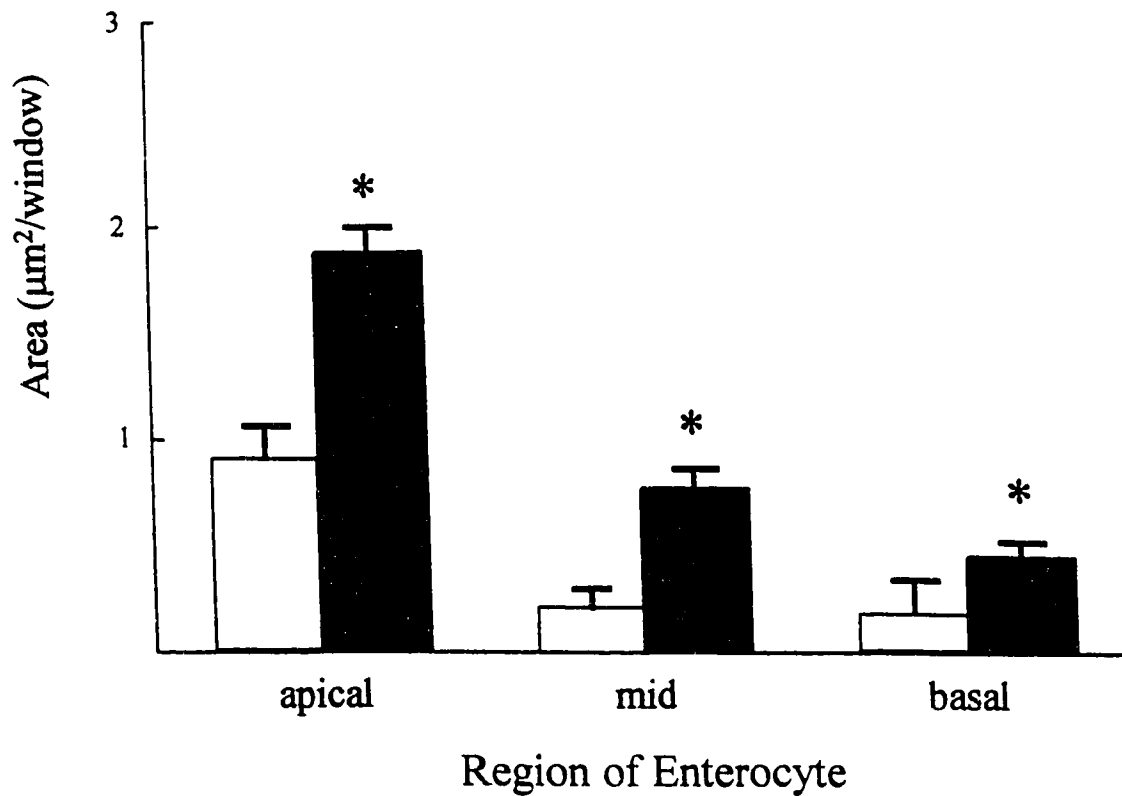


Figure 5.3 Effect of stress on uptake of horseradish peroxidase into villus enterocytes. Area of HRP product-filled endosomes in enterocytes of tissues fixed 120 min after the addition of HRP. Bars indicate endosomal area positive for HRP activity/window (expressed as means \pm SEM; n is 30 windows) for $25 \times 30 \mu\text{m}^2$ windows in the apical, mid, and basal regions of colonic enterocytes; 30 windows were examined from each group with 4 rats per group. Endosome area was significantly increased in tissues from stressed rats (solid bars) compared to controls (open bars) for all three regions of the enterocytes. * $p < 0.001$ for apical region, * $p = 0.002$ for mid region, and * $p = 0.005$ for basal region.

by 96.4%, and completely preventing the increased flux of HRP (diminished by 100%), while there was a trend for partial recovery of the stress-induced increase in baseline conductance, it was diminished by 40.8%. This measure in the CRF antagonist group was not significantly different from the stress group (Table 5.2). Injection of Wistar Kyoto rats with CRF (ip) 4 h before study mimicked the effects of stress (Table 5.2). There were significant increases in both baseline I_{sc} and conductance as well as a significant increase in flux of HRP. These measures from the CRF group are not significantly different compared to the stressed group.

TABLE 5.2 Mediators of the Colonic Mucosal Stress Responses in Wistar Kyoto Rats

	I_{sc} ($\mu\text{A}/\text{cm}^2$)	G (mS/cm^2)	HRP flux ($\text{pmol}/\text{h}/\text{cm}^2$)
control	59.8 \pm 6.9	14.8 \pm 1.2	6.4 \pm 1.9
stress	98.6 \pm 8.9 *	22.4 \pm 2.9 *	16.6 \pm 3.4 *
stress & atropine	83.7 \pm 8.9	19.9 \pm 2.5	12.7 \pm 3.7
stress & $\alpha\text{h-CRF}_{9-41}$	61.2 \pm 9.0 #	19.3 \pm 3.2	6.3 \pm 3.3 #
CRF	97.5 \pm 14.6 *	23.0 \pm 3.3 *	18.3 \pm 5.0 *

Baseline short-circuit current (I_{sc}) and baseline conductance (G) measures 20 min after mounting colonic mucosal segments (from Wistar Kyoto rats) on Ussing chambers and the luminal to serosal flux of HRP across the tissue. Stressed rats had significant differences from control values for baseline I_{sc} ($p=0.005$), baseline G ($p=0.036$) and HRP flux ($p=0.029$). Atropine pretreatment partially corrected the stress induced increases in the tissues' responses to stress. Pretreatment of the CRF antagonist α helical-CRF₉₋₄₁ partially corrected the conductance measure while fully correcting the stress-induced increases in baseline I_{sc} and flux of HRP. An ip administration of CRF mimicked the stress responses with baseline I_{sc} ($p=0.042$), baseline G ($p=0.041$) and HRP flux ($p=0.041$), all being significantly different from control measures. Values shown represent the means \pm SEM, $n=5-6$ rats with 2-4 tissues averaged for each rat; * indicates a significant difference from control values ($p<0.05$), # indicates a significant difference from stressed values ($p<0.05$).

5.4 DISCUSSION

We have elaborated on our previous findings [13,18,19] showing that stress-induced pathophysiology of intestinal mucosa not only occurs in the jejunum, but also in the colon. In the stress susceptible Wistar Kyoto rats acute stress caused increased baseline I_{sc} and compromised epithelial barrier as indicated by the increased baseline conductance, flux of the small molecular probe, fMLP and the macromolecule, HRP. The evidence also indicates that at least some of these effects of stress are due to the action of CRF released in the periphery.

The stress-induced increase in baseline I_{sc} was more evident in the parental Wistar rat strain than in Wistar Kyoto rats (234% vs. 128% of control). Wistar rats have been used to document stress-induced perturbations in ion transport abnormalities, as observed in this study, and increased mucous production [6] or increased colonic transit [16]. These colonic responses may be considered to be protective mechanisms for limiting noxious material gaining access to the mucosa by flushing such material away out of the lumen. However, Wistar rats did not exhibit stress-induced increases in permeability. On the other hand, stressed Wistar Kyoto rats demonstrated a significantly compromised epithelial barrier compared to controls. Colonic epithelium from stressed Wistar Kyoto rats was significantly more permeable to ions, small molecules including fMLP, and macromolecules up to the size of HRP (40kD). We used the biologically relevant bacterial tripeptide, fMLP, as a probe to examine if stress caused an increased flux across the colonic epithelium of this proinflammatory mediator. fMLP activates neutrophils, macrophages and to a lesser extent eosinophils; in conjunction with lipopolysaccharide, another bacterial product, fMLP is an extraordinarily potent activator of resident immune cells in the colonic mucosa [3,12,21,22].

We have previously shown [13] that stress caused a dramatic increase in the flux of HRP across jejunal epithelia increasing transcellular transport by escalating endosomal uptake and inducing paracellular movement past tight junctions. In this study, we showed that colon also responds to stress by significantly increasing the luminal to serosal movement of HRP. Electron microscopic analysis revealed occasional evidence of paracellular transport. The qualitative and quantitative analysis indicated that by far the most predominant route of HRP transport across the colonic epithelia was via transcellular endosomes. Stress caused the increased flux of intact protein through the enterocytes as determined by the flux data. Compared to controls, enterocytes from stressed rats showed significantly greater areas of HRP positive endosomes in all three cellular regions, apical, mid, and basal.

Exploring the mechanisms responsible for the stress-induced pathophysiology of the gastrointestinal tract, we previously reported the importance of cholinergic nerves mediating the responses to stress in the jejunum [18]. We used the same dose of atropine as that used in the previous study which completely blocked the stress responses in the jejunum. In this study the atropine did not significantly alleviate the effects of stress in the colonic mucosa, however, there was the trend that atropine treatment partially reduced the stress responses. We used a relatively high dose of atropine since Wistar Kyoto rats are hypercholinergic and the intestinal mucosa is sensitive to acetylcholine compared to the parental Wistar rats [18]. Other cells and mediators are definitely involved in mediating the responses to stress in the colon.

Central CRF has been shown to mediate many stress-induced responses in the gastrointestinal tract, including stress-induced gastric ulceration, and intestinal transit

abnormalities [5,10,16,23]. In this study, we showed that CRF administered ip mimicked the mucosal responses to stress in the colon. Since CRF can cross the blood brain barrier, it was not clear if its effects were due to peripheral and/or central action. However, the peripheral, ip, administration of the CRF antagonist α -helical CRF₉₋₄₁, which does not cross the blood brain barrier, blocked most of the stress effects in the colon. These findings indicate that peripheral CRF mediated the colonic mucosal stress responses. Others have also shown peripheral antagonism of CRF inhibiting other intestinal stress responses [6]. Our results do not rule out a role for central CRF in mediating the stress responses, but highlight the importance of peripheral CRF activity.

In summary our key finding in this study is that in contrast to Wistar rats Wistar Kyoto rats have a permeability defect in their colonic epithelium that is exposed when the rats are stressed. Evidence has been reported that a subpopulation of relatives of IBD patients may have increased baseline intestinal permeability [15] suggesting a genetic predisposition for an epithelial barrier defect. However, this is a controversial area of clinical research. Our stress model using Wistar Kyoto rats may lead to a greater understanding of the mechanisms by which relapses are initiated and how stress contributes to intestinal inflammation.

The authors acknowledge the expert technical assistance of Ms. M. Benjamin and the staff of the Electron Microscopy Unit, Faculty of Health Sciences, McMaster University. This research was supported by a grant from the Medical Research Council of Canada.

5.5 REFERENCES

1. Almy T.P., F. Kern Jr., and M. Tulin. Alterations in colonic function in man under stress: experimental production of sigmoid spasm in healthy persons. *Gastroenterology* 12:425-436, 1949.
2. Andrianopoulos G., C.T. Bombeck, and L.M. Nyhus. Influence of maturation on activity-stress related pathology in the rat colon. *Physiol. & Behav.* 36:1105-10, 1986.
3. Bach, M.K. and J.R. Brashler. FMLP is a potent activator of guinea-pig eosinophils but its activity is dependent on the prior overnight *in vitro* culture of the cells (facilitation). *Immunology* 75: 680-687, 1992.
4. Bijlsma P.B., A.J. Kiliaan, G. Scholten, M. Heyman, J.A. Groot, and J.A.J.M. Taminiaw. Carbachol but not forskolin can increase mucosal to serosal transport of intact protein in rat ileum *in vitro*. *Am. J. Physiol.* 271:G147-G155, 1996.
5. Bueno L., S.M. Collins, and J.L. Junien. *Stress and Digestive Motility*. London; John Libby Publishers; 1989.
6. Castagliuolo I., J.T. LaMont, B. Qiu, S.M. Fleming, K.R. Bhaskar, S.T. Nikulasson, C. Kornetsky, and C. Pothoulakis. Acute stress causes mucin release from rat colon: role of corticotropin releasing factor and mast cells. *Am. J. Physiol.* 271: G884-G892, 1996.
7. Crowe S.E. and M.H. Perdue. Gastrointestinal food hypersensitivity: basic mechanisms of pathophysiology. *Gastroenterology* 103:1075-1095, 1992.
8. Duffy L.C., M.A. Zielezny, J.R. Marshall, T.E. Byers, M.M. Weiser, J.F. Phillips, B.M. Calkins, P.L. Ogra and S. Graham. Relevance of major stress events as an indicator of disease activity prevalence in inflammatory bowel disease. *Behav. Med.* 17:101-110, 1991.
9. Empey, L.R. and R.N. Fedorak. Effect of misoprostol in preventing stress-induced intestinal fluid secretion in rats. *Prostaglandins Leukotrienes Essent. Fatty Acids.* 38: 43-8, 1989.
10. Glavin G.B. Restraint ulcer: history, current research and future implications. *Brain Res. Bull.* 5:51-58, 1980.

11. Graham R.C. and M.J. Karnovsky. Glomerular permeability: ultrastructural cytochemical studies using peroxidases as protein tracers. *J. Exp. Med.* 124:1123-1134, 1966.
12. Karlsson, A., M. Markfjall, N. Stromberg and C. Dahlgren. *Escherichia coli*-induced activation of neutrophil NADPH-oxidase: lipopolysaccharide and formylated peptides act synergistically to induce release of reactive oxygen metabolites. *Infection & Immunity* 63: 4606-4612, 1995.
13. Kiliaan, A.J., P.R. Saunders, P.B. Bijlsma, C. Berin, J.A. Taminiau, J.A. Groot and M.H. Perdue. Stress stimulates transepithelial macromolecular uptake in rat jejunum. *Am. J. Physiol.* in press. 1998.
14. Lim, D.K., I.B. Hoskins, R.W. Rockhold and I.K. Ho. Comparative studies of muscarinic and dopamine receptors in three strains of rat. *Eur. J. Pharmacol.* 165: 279-87, 1989.
15. May G.R., L.R. Sutherland and J.B. Meddings. Is small intestinal permeability really increased in relatives of patients with Crohn's disease? *Gastroenterology* 104:1627-32, 1993.
16. Monnikes, H., B.G. Schmidt, H.E. Raybould and Y. Taché. CRF in the paraventricular nucleus mediates gastric and colonic motor response to restraint stress. *Am. J. Physiol.* 262: G137-43, 1992.
17. Pare W.P. Stress ulcer susceptibility and depression in Wistar Kyoto (WKY) rats. *Physiol. Behav.* 46:993-998, 1989.
18. Saunders P.R., N.P.M. Hanssen, D.S. Chandraratne and M.H. Perdue. Cholinergic nerves mediate stress-induced intestinal transport abnormalities in Wistar Kyoto rats. *Am. J. Physiol.*, 237: G486-G490, 1997.
19. Saunders P.R., U. Kosecka, D.M. McKay and M.H. Perdue. Acute stressors stimulate ion secretion and increase epithelial permeability in rat intestine. *Am. J. Physiol.* 267: G794-G799, 1994.
20. Sullivan A. Psychogenic factors in ulcerative colitis. *Am. J. Dig. Dis.* 2:651-656, 1935.
21. Surette, M.E., M. Nadeau, P. Borgeat and J. Gosselin. Priming of human peripheral blood mononuclear cells with lipopolysaccharides for enhanced arachidonic acid release and leukotriene synthesis. *J. Leukoc. Biol.* 59: 709-715, 1996.

22. von Ritter, C., M.B. Grisham, M. Hollwarth, W. Inauen and D.N. Granger. Neutrophil-derived oxidants mediate formyl-methionyl-leucyl-phenylalanine-induced increases in mucosal permeability in rats. *Gastroenterology*: 97: 778-780, 1989.
23. Wittman T., F. Crenner, F. Angel, L. Hanusz, C. Ringwald and J.F. Grenier. Long duration stress. Immediate and late effects on small and large bowel motility in rat. *Dig. Dis. Sci.* 35: 495-500, 1990.
24. Young S.J., D.H. Alpers, C.C. Norland and R.A. Woodruff. Psychiatric illness and the irritable bowel syndrome. Practical implications for the primary physician. *Gastroenterology* 70:162-166, 1976.

Chapter 6

DISCUSSION

NOTE TO USERS

**Page(s) missing in number only; text follows.
Microfilmed as received.**

140

This reproduction is the best copy available.

UMI

DISCUSSION

6.1 SUMMARY

Many studies have examined stress-induced gastric ulceration and stress-induced alterations in gastrointestinal motility; however, few studies have examined the effects of stress on intestinal epithelial physiology. The purpose of my studies was to define stress-induced changes in the intestinal epithelium and the mechanisms involved in the epithelial responses.

6.1.1 Objective 1: To Define Stress-Induced Pathophysiology of Intestinal Epithelium.

We used stress-susceptible Wistar Kyoto rats as subjects for our experiments. For the initial study, rats were stressed by restraint at either 22°C (room temperature) or at 8°C for 4 hours. All subsequent studies involved 2h of cold-restraint stress followed by 2h of rest at room temperature. Jejunal tissues from stressed or control rats were removed and parameters of physiology were studied in Ussing chambers. Acute stress caused a significant increase in the baseline short-circuit current, a measure of net active ion transport by the epithelium. We showed that this increase in baseline short-circuit current in tissues from stressed rats was due to chloride ion secretion, since such tissues when bathed in buffer in which chloride was replaced by isethionate, demonstrated the same baseline short-circuit current as control tissues. In addition, fluxes of radiolabelled sodium and chloride ions showed that the normal

mucosal-to-serosal absorption of chloride was reversed and that there was serosal-to-mucosal secretion of chloride in tissues from stressed rats; the pattern for sodium remained unaffected by stress. The same study also revealed that tissues from stressed rats had reduced short-circuit current responses to electrical transmural stimulation of the tissue. When we tested the secretory ability of the epithelium to bethanechol (an analogue of acetylcholine) and to vasoactive intestinal peptide, tissues from stressed rats had the same or greater response compared with tissues from control rats. This indicated that the reduced response to transmural stimulation was due, at least in part, to impaired neural activity in the intestinal mucosa from stressed rats. This suggested that local enteric nerves were involved in mediating the stress responses in the tissue. We showed that stress also caused a significant increase in the baseline short-circuit current of distal colonic epithelium. Our results confirm and extend those of earlier investigations, showing that stress caused water secretion and disturbances in ion transport in human jejunum and water secretion in the distal small intestine and colon of rats (Barclay & Turnberg, 1987, 1988a; 1988b; Empey & Fedorak, 1989).

The barrier property of intestinal epithelium was also dramatically altered by stress. Jejunal tissues from stressed rats had increased baseline conductance measurements (an indication of increased ionic permeability) compared with tissues from control rats. Epithelial permeability to the small inert probes, mannitol and Cr-EDTA, was found to be significantly greater in tissues from stressed rats versus controls. Colonic tissues from stressed Wistar Kyoto rats also had significantly elevated baseline conductance measures as well as significantly higher fluxes for the bacterial tripeptide, fMLP. This evidence suggested that the epithelial tight junctions were leakier, since ions and these small probes typically cross the

epithelium via the paracellular route.

We wanted to know if stress could compromise the intestinal epithelial barrier to much larger molecules, for example, potentially antigenic material. We found that both the jejunum and the distal colon from stressed rats had significantly greater luminal to serosal fluxes of the protein, horseradish peroxidase. Electron microscopy revealed that there was increased endocytosis and intracellular transport of intact horseradish peroxidase in both the jejunum and colon of stressed rats. In addition, in the jejunum stress stimulated paracellular transport of horseradish peroxidase through the tight junctions, whereas this finding was never observed in control tissues. Previous investigations have shown that traumatic stressors can compromise the barrier property of the intestinal epithelium (Rhodes & Karnofsky, 1971; Carter et al., 1990), however, this is the first study to show that a mild stressor can also cause the epithelium of the small bowel and the colon to be more permeable to large as well as small molecules. Since our findings, Meddings et al. (1997) have also shown in a preliminary report that mild stress can cause an increase in the epithelial permeability for small sugar molecules, predominantly along the proximal gastrointestinal tract.

6.1.2 Objective 2: To Explore Mechanisms Responsible for Stress-Induced Pathophysiology of Intestinal Epithelium.

Our observations in the initial study indicating impaired neural activity in jejunal tissues from stressed Wistar Kyoto rats suggested that local enteric nerves were instrumental in mediating the epithelial stress responses. Acetylcholine is a prominent neurotransmitter in the enteric nervous system that regulates several key epithelial functions and has been shown

to mediate other gastrointestinal stress responses such as gastric ulceration and altered intestinal motility (Cooke & Reddix, 1994; Bijlsma et al., 1996; Gaton et al., 1993). Pretreatment of Wistar Kyoto rats with atropine sulfate (via ip injection) prior to stress prevented the stress-induced physiological responses of the jejunal epithelium: the baseline short-circuit current was not significantly different from control readings, the baseline conductance measurements were at control values, as were the fluxes of Cr-EDTA and horseradish peroxidase. In addition, unlike results in untreated stressed rats, electron microscopy showed transcellular transport of this protein similar to control levels and there was no evidence of paracellular transport of horseradish peroxidase. To examine whether peripheral or central cholinergic receptors were involved, we pretreated rats with atropine methyl nitrate, a quaternary salt that does not cross the blood brain barrier. The same findings were obtained with atropine methyl nitrate as with atropine sulfate, suggesting that acetylcholine released in the tissues and acting on muscarinic receptors was responsible for mediating the stress effects. However, atropine sulfate treatment could only partially alleviate the stress-induced epithelial responses in the distal colon.

Other mediators may be involved in the stress-induced responses in the distal colonic epithelium. Following leads from other studies (Monnikes et al., 1992; Castagliuolo et al., 1996) we examined the role of corticotropin releasing factor. Pretreatment (via ip injection) of Wistar Kyoto rats with the corticotropin antagonist, α -helical CRF₉₋₄₁ (a form that does not cross blood brain barrier) dramatically reduced the stress-induced responses in colonic tissues: the baseline short-circuit current was comparable to control values, the baseline conductance had partially recovered, while the horseradish peroxidase flux had normalized. In addition,

peripheral (ip injection) administration of corticotropin-releasing factor to naive Wistar Kyoto rats mimicked all the stress-induced pathophysiology in colonic tissue. Taken together, these two findings strongly implicate peripheral corticotropin-releasing factor in the pathway mediating the altered colonic physiology caused by acute stress. The source of the local corticotropin releasing factor is unclear from our studies, but may be released from either local enteric nerves, enteroendocrine cells, or even local immunocytes such as macrophages (Crofford et al., 1995). Although central corticotropin releasing factor activity mediating gastrointestinal stress responses has been well characterized (Monnikes et al., 1992), peripheral corticotropin releasing factor activity has also been shown to mediate stress-induced mucus secretion in the rat colon (Castagliuolo et al., 1996).

We compared the stress-induced intestinal epithelial responses in the stress susceptible rat strain, Wistar Kyoto (Pare, 1989 b), with those in the parental Wistar rat strain. Both strains showed significant increases in baseline short-circuit and conductance in jejunal tissues in response to stress. However, the stress-induced increases were far greater in Wistar Kyoto rats, particularly the elevation of baseline conductance. The only exception was found in colonic tissues where Wistar rats showed a greater increase in the baseline short-circuit current compared to Wistar Kyoto rats. However, colonic tissue from Wistar rats showed no impaired epithelial barrier properties. The baseline conductance of tissues from stressed Wistar rats was normal along with fMLP and horseradish peroxidase fluxes, whereas all three measures were significantly increased in response to stress in tissues from stressed Wistar Kyoto rats. We identified that the intestinal mucosa of Wistar Kyoto rats has less cholinesterase activity than that of Wistars. Wistar Kyoto rats also have less cholinesterase

activity in blood compared to Wistar and Sprague Dawley rats (Lim et al., 1989). The consequence may be that Wistar Kyoto rats are hypercholinergic compared to Wistar rats. Tissues from Wistar Kyoto rats were more responsive to exogenously administered acetylcholine compared with tissues from Wistar rats. The hypercholinergic state may contribute to the Wistar Kyoto's susceptibility to stress and may explain the reason that a relatively high dose of atropine was required to inhibit the epithelial stress responses.

Overall, our studies showed that Wistar Kyoto rats developed more extreme intestinal abnormalities in response to stress than did Wistar rats. These findings indicate that genetic factors are involved in stress susceptibility. I speculate that a genetic predisposition to stress may be caused by an imbalance in activity of parasympathetic and sympathetic nerves resulting in a hypercholinergic state that increases gut permeability in response to relatively mild stress.

The colonic mucosa of Wistar rats also responded to stress with ion secretion. Wistar rats have been used to document increases in colonic mucus production (Castagliuolo et al., 1996) and increased colonic transit (Monnikes et al., 1992) after short periods of stress. These responses are generally considered protective mechanisms for inhibiting noxious material from gaining access to the mucosa by flushing the offending material away. The noxious material may be ingested proteins, bacterial products or even bacteria themselves. These physiological responses are appropriate for an individual facing an insult from the gut lumen. The physiological responses to mild stressors will not manifest as a pathological state. However, the compromised intestinal barrier in stressed Wistar Kyoto rats may not be able to prevent noxious luminal material from crossing the very permeable epithelium and reacting

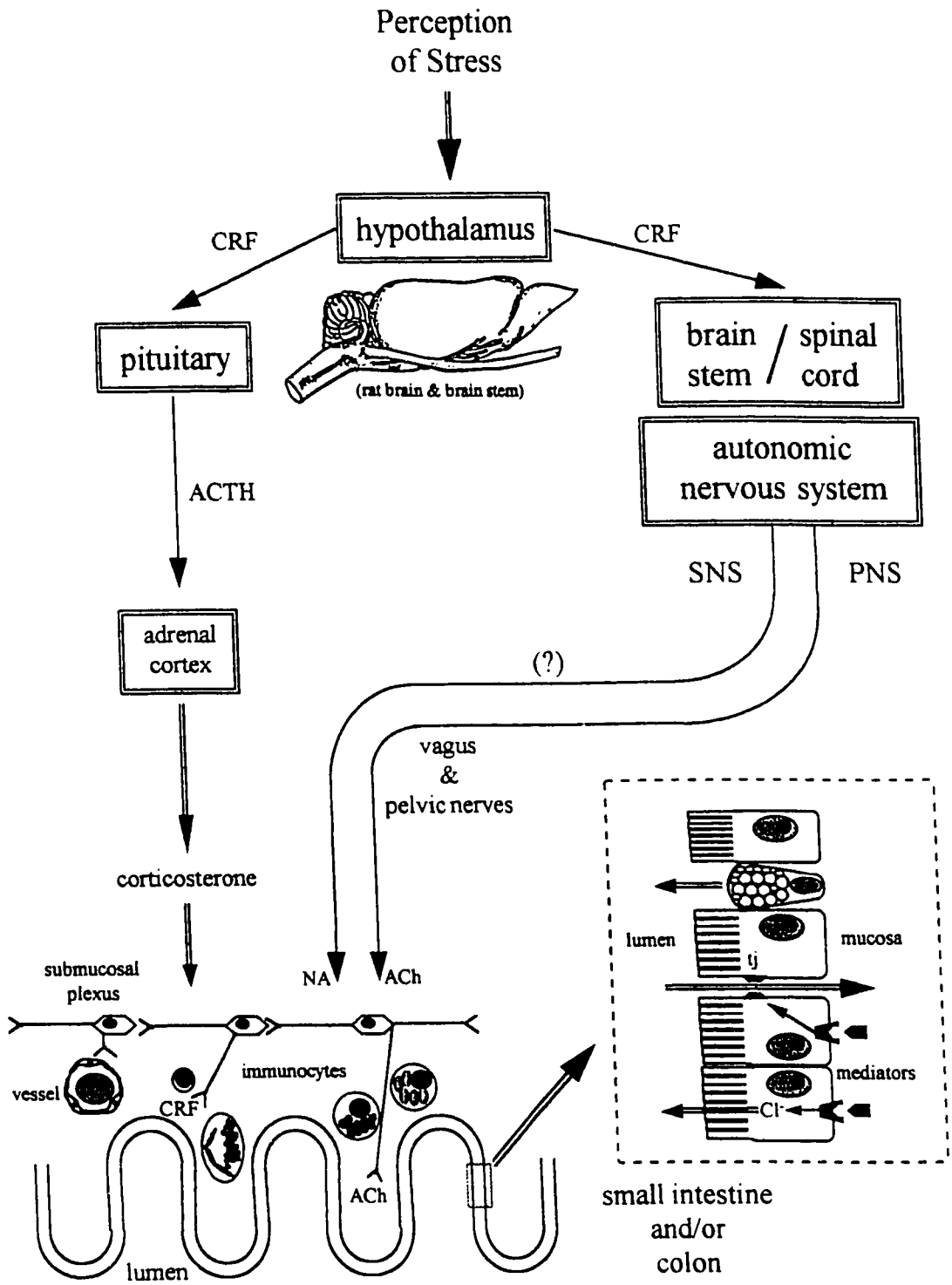
with local immunocytes residing in the mucosa. A similar response may contribute to symptoms in humans suffering with intestinal diseases. If so, stressed Wistar Kyoto rats are a very interesting model to further study the role of stress in inflammatory bowel disease and irritable bowel syndrome.

We have clearly shown that stress can disturb the normal intestinal physiology in genetically susceptible rats. Our model appears to be an excellent one to gain a clearer understanding of the mechanisms by which stress mediates clinical symptoms in humans and intestinal pathology.

6.2 CLINICAL SIGNIFICANCE & SPECULATIONS

My studies suggest that stress contributes to relapses in gastrointestinal diseases by compromising the epithelial barrier. In addition to anecdotal evidence linking stress to intestinal symptoms, there is clinical evidence that humans with inflammatory bowel diseases are at much greater risk for inflammatory relapses after experiencing stressful life events (Duffy et al., 1991). Stressed Wistar Kyoto rats, a susceptible strain, demonstrated significantly increased epithelial permeability compared to controls. The colonic epithelium from stressed rats was more permeable to potentially active small and large molecules, including the bacterial tripeptide, fMLP, and to an antigenic sized protein. My findings lead to the hypothesis that when individuals who react to their luminal antigens, and who are also genetically susceptible to stress, are exposed to stressful situations for sufficient periods of time, their epithelium becomes leaky, resulting in uptake of molecules from the lumen. Stimulation of local mucosal immunocytes results in gross inflammation at specific sites in the

Figure 6.1 Schema of mechanisms mediating the stress-induced epithelial pathophysiology in the jejunum and colon. When the rat perceives a stressor, the hypothalamus is activated and releases corticotropin-releasing factor (CRF). CRF signals the pituitary to release adrenocortical hormone (ACTH) which in turn signals the adrenal cortex to produce and release corticosterone into the blood. Corticosterone has many activities on several organs throughout the body including the gastrointestinal tract. CRF from the hypothalamus also signals brain stem nuclei stimulating the autonomic nervous system. The sympathetic nervous system (SNS) is activated first followed by a rebound activity of the parasympathetic nervous system (PNS). It is not clear whether the preceding SNS activity releasing noradrenaline (NA) is necessary for the epithelial pathophysiology that occurs with involvement of cholinergic receptors. Stress may induce acetylcholine (ACh) release at various points. ACh may be released from the PNS stimulating the submucosal plexus in the gut wall. Local immunocytes such as mast cells, eosinophils, macrophages and lymphocytes may also be involved in mediating the stress responses. In the colon, peripheral CRF is involved in mediating the stress responses. The source of the CRF is not clear. It may be from enteric nerves, enteroendocrine cells in the epithelium, or even local immunocytes. Whatever final mediators are involved, the epithelium ultimately responds to the stress by secreting chloride and mucus and opening tight junctions, allowing luminal material to enter the mucosa.



intestine. Recent studies have shown that mucosal immunocytes obtained from patients with Crohn's disease react specifically to microbes isolated from the gut of the same individuals, while immunocytes from healthy humans do not (Duchmann et al., 1995). In addition, memory lymphocytes expressing the leukocyte common antigen (CD45RO) are present in greater quantities in the peripheral blood of patients with Crohn's disease (along with their first degree relatives) compared to healthy controls. The presence of CD45RO⁺ lymphocytes correlates with increased intestinal permeability (Yacyshyn & Meddings, 1995). Although there is still controversy (Munkholm et al., 1994), relatives of inflammatory bowel disease patients may have increased baseline intestinal permeability (Hollander et al., 1986), suggesting a possible genetic predisposition for this defect. It is likely that a subpopulation (~10%) of first degree relatives of inflammatory bowel disease patients have increased epithelial permeability (May et al., 1993). It would be interesting to examine whether these relatives show greater increased permeability in response to controlled experimental stressors compared to control subjects. This has not been reported. However, approximately 35% of first degree relatives of patients with Crohn's disease demonstrated a hyperresponsive reaction involving increased intestinal epithelial permeability following a challenge with acetylsalicylic acid, whereas there were no hyperresponders in the control population (Hilsden et al., 1996).

Exploring the mechanisms responsible for the stress-induced pathophysiology of the gastrointestinal tract, we report the importance of cholinergic nerves mediating the responses to stress in the jejunum. We also demonstrated that Wistar Kyoto rats are hypercholinergic, due in part to decreased tissue cholinesterase activity. In the clinical setting, decreased plasma

cholinesterase levels are the best indicator of inflammatory severity, as compared with other standard laboratory tests, in Crohn's patients suffering from an active relapse (Tromm et al., 1992) (endoscopic observation and biopsy was the gold standard for evaluating the severity of inflammation). Therefore, cholinergic hypersensitivity and stress susceptibility may be two key characteristics that predispose individuals to contracting inflammatory bowel diseases.

My experiments also implicate peripheral corticotropin releasing factor in mediating the stress responses. Corticotropin releasing factor has been measured in synovial fluid of patients with rheumatoid arthritis and in a rat model of experimental arthritis. Peripheral corticotropin releasing factor also has proinflammatory activity (Crofford et al., 1995). Local production of corticotropin releasing factor has been observed in the caecal mucosa of rats with experimental intestinal inflammation as well as in the colonic mucosa of humans suffering from active ulcerative colitis (van Tol et al., 1996; Kawahito et al., 1995). I speculate that therapies targeted at inhibiting negative peripheral activity of corticotropin releasing factor may prove useful for the treatment of intestinal inflammation.

6.3 FUTURE DIRECTIONS

I advocate that future research using this model of stress-induced pathophysiology of intestinal mucosa be conducted along two main lines. The first is to further elaborate on the mechanisms responsible for the stress responses in the intestinal mucosa. The second is to develop animal models involving stress that reflect critical components of human intestinal inflammatory disease.

6.3.1 Further Mechanisms Involved in the Stress Responses

In our experiments, we implicated peripheral acetylcholine and peripheral corticotropin releasing factor activity in mediating the stress responses in the intestinal mucosa. However, this does not mean that these mediators of the stress responses acted directly on the epithelium and acted alone, especially in the case of colonic stress responses when these agents only partially inhibited the responses. Another piece of evidence suggesting that these agents do not act alone is the fact that atropine completely blocked the stress responses in the jejunum, providing it was given prior to the rats being stressed. Atropine was ineffective at inhibiting intestinal changes if it was given to the rats after being stressed suggesting that acetylcholine indirectly contributed to the production of the epithelial responses. Other studies examining gastrointestinal responses to stress, such as increased transit, permeability, mucus production or ulceration, suggest the involvement of mast cells and their mediators, such as histamine (possibly even the protease RMCP II), eicosanoids such as leukotrienes and thromboxane (prostaglandin E is protective), glucocorticoids, substance P, adrenergic nerves, cholinergic nicotinic activity, opioids (β -endorphin), microcirculation of the gastrointestinal mucosa and central corticotropin releasing factor and thyrotropin releasing hormone (Basso et al., 1982; Castagliuolo et al., 1996; Garrick, 1990; Gaton et al., 1993; Kitagawa et al., 1984; Kitajima et al., 1991; Lenz, 1989; Meddings et al., 1997; Monnikes et al., 1992; Orlando et al., 1985; Ray et al., 1987; Santos et al., 1998). Further experiments are required to verify if any or all of these mediators are responsible for the stress-induced intestinal epithelial responses I recorded. Such experiments might also provide the sequence of mediator involvement that eventually results in the intestinal changes.

We have observed differences in severity of intestinal epithelial responses to stress between two related strains of rats. We have attributed these differences to a genetic predisposition for hypercholinergic activity due to reduced cholinesterase levels in the intestinal mucosa of Wistar Kyoto rats. It may be relevant to examine other aspects of the cholinergic response in these rats. The Wistar Kyoto strain was selectively bred for normal tensile responses to be the control strain for the Spontaneously Hypertensive rats. Wistar Kyoto rats also may have a disturbed hypothalamic-pituitary axis that may contribute to their stress susceptibility. There is evidence that these rats have an impaired negative feedback mechanism regulating the hypothalamic-pituitary stress hormones as well as an insensitivity to glucocorticoids (Redei et al., 1994).

6.3.2 Stress Mediated Intestinal Inflammation Models

The development of animal models of intestinal inflammation involving stress as a contributing factor may be useful in explaining relapses in inflammatory bowel diseases. It may be that sensitized individuals who are genetically stress-susceptible develop a defect of the epithelial barrier, allowing a greater influx into the mucosa of potentially reactive material from the intestinal lumen. Noxious agents from the lumen may then react with local and recruited immunocytes causing gross macroscopic inflammation. It may be possible to utilize this hypothesis to develop an appropriate model. Bacterial antigens can be produced by culturing from the colonic lumen of normally housed Wistar Kyoto rats. A cocktail of these bacterial antigens can be injected, with appropriate adjuvants, into rats to sensitize them to their luminal contents, as has been demonstrated for patients with inflammatory bowel disease

(Duchmann et al., 1995). Using different adjuvants (such as incomplete Freund's, alum or pertussis vaccine), we may be able to bias the immune response to either a T-helper type 1 or type 2 response. It has been suggested that Crohn's disease involves type 1 T cells and cytokines (IFN γ and Il-2), while the inflammatory reaction in ulcerative colitis utilizes type 2 T cells and cytokines (Il-4, Il-5 and Il-10) (Sartor, 1995). Therefore, we may be able to create more relevant models in the rats. Sensitized rats can be subjected to a number of different mild stressors, rather than a single acute episode of stress, mimicking the chronic stress situation in humans. The stressors should involve a psychological component instilling fear, anxiety or frustration (Johnson et al., 1992), shown to be important to intestinal pathophysiology. Such stressors can include reversing the rat's light/dark cycle, flashing lights, new noises, motion, restraint, exposure to cold or heat, exposure to water, exercise, pain and/or fasting. It will be important to record behavioural changes such as reduced activity and food intake, as well as signs of intestinal dysfunction such as diarrhea, to predict the best time to study bowel histology and physiology. Limited animal models have been defined along these lines. Wood (1996) reported that the spontaneous ulcerative colitis observed in cotton top tamarins was associated with the stress of captivity. In these animals intestinal injury was absent from Thiry-Vella loops of colon isolated from the rest of the colon and kept clean, i.e. bacteria free. When fecal contents were returned to the isolated segments, the colitic injury reappeared. In addition, stress-induced recurrence of inflammation in DNB or TNB (di or trinitrobenzene) treated rats and mice occurred only when the hapten was present in the colon (Qiu et al., 1997; Jacobson et al., 1997).

6.4 OVERALL SIGNIFICANCE

Understanding the mechanisms of stress-induced epithelial pathophysiology may assist in the development of novel pharmaceutical agents that protect the epithelium from stress-induced injury. If we can predict the stress-induced prepathological state in humans, then drugs that repair the compromised epithelial barrier may be administered in time to prevent any inflammatory relapses. This research is a beginning for future studies that may lead to general insights into the effects of stress on epithelial cells. In the future, such research may result in the understanding of the role of stress in diseases at other mucosal sites.

NOTE TO USERS

**Page(s) missing in number only; text follows.
Microfilmed as received.**

156

This reproduction is the best copy available.

UMI

APPENDIX A

NOTE TO USERS

**Page(s) missing in number only; text follows.
Microfilmed as received.**

158

This reproduction is the best copy available.

UMI

APPENDIX A:

A.1 EXTENDED IDEAS REGARDING STRESS

A.1.1 General Adaptation Theory

When an animal is stressed a number of physiological responses occur. The alarm reaction is the animal's typical immediate response to acute stress. If the animal is stressed repeatedly (with the same stressor) the responses will not be as severe as they were following the initial stress. The General Adaptation Theory postulates that energy is consumed in adaptation to the environmental stressor, which an organism can now predict the occurrence of, so its internal environment is not as severely altered, this is known as the resistance period. The animal does not have an unlimited supply of energy and if the stress is constantly repeated in sufficient time the energy will be depleted, then severe pathology will result and ultimately death, this is regarded as the period of exhaustion (Selye, 1946).

A.1.2 Defining Stress (Inducer of a Prepathological State)

We are left with the very general definition that stress is the body's response when homeostasis is threatened (Moberg, 1985; Stratakis & Chrousos, 1995). Most often, the responses by the body are appropriate and necessary for survival. Most researchers are interested in the maladaptive stress responses that eventually results in disease, ie. when the responses to stress are either inadequate or excessive and as a result lead to altered

physiology that can cause pathology (Chrousos & Gold, 1995). Some researchers, instrumental in the field of stress, believe that a single unifying objective definition of stress (ie symptoms or responses) is not possible (Overmier, 1988).

Is it possible to assess an animal's well being in response to stress? Examining only secreted neuroendocrine mediators is not sufficient. Although, it is well recognized that there are critical neuroendocrine mediators regulating the responses to stress, just their levels or their change in levels does not provide an adequate answer to how stressed the animal might be or will it develop a pathology or not. To know if an animal is stressed a number of parameters needs to be examined developing a pattern of the stress response. The impact of the neuroendocrine systems during stress responses on such functions as reproduction, growth, metabolism or the impact on the cardiovascular, gastrointestinal or immune systems gives a better idea of an animals state of well being (Stratakis & Chrousos, 1995). A key goal is to understand how stress contributes to disease. Just studying pathology, resulting from stress, may not reveal the component of the stress response that ultimately caused the pathology. Plus, in many cases we want to prevent the pathology from occurring, so we need some indicators that an animal is in a state of poor 'well-being' and is at risk for developing pathology before pathology can actually be documented. Moberg (1985) addresses this issue by suggesting we need to know the physiological responses that occur in response to stress and ultimately result in pathology. By evaluating these physiological responses in a prepathological state we may determine how well is an animal, ie how stressed is an animal. Moberg suggests that being in a prepathological state does not guarantee pathology, an animal may recover, but the longer an animal is in a prepathological state the more at risk the

animal is for developing pathology. Of course different individuals will have different thresholds for different stressors that cause them to be in a prepathological state.

A.1.3 Defining My Stress Model

I do not provide a universal definition of stress. I used a common stressor (cold restraint stress) in gastrointestinal research to examine what effects such a stressor might have on normal intestinal epithelial physiology. I saw a pattern of epithelial pathophysiology which I describe throughout the thesis. I was interested in this pattern of responses, so I used animals that responded with this pattern of epithelial pathophysiology following stress. I do not believe that if an animal did not demonstrate these epithelial responses after being subjected to the stressor that these animals were not stressed, however, I am interested in those animals that did respond to the stressor with epithelial pathophysiology and for the practical purposes of this thesis I consider those animals as being stressed. I did not routinely use an independent measure of stress, I concentrated on the intestinal epithelial response as a possible prepathological state some animals may experience, that if exposed to for a sufficient length of time the animal would suffer from intestinal pathology.

I refer to the procedure I use as acute stress. My procedure lasts 4 hours and I am well past the immediate effects of the sympathetic nervous system response (fight or flight) to the stress (a common neuroendocrine response to stress that may occur within seconds of experiencing stress). Because of the timing of this stress protocol I have been questioned if this stress is actually acute stress. I believe my procedure is acute stress because I view chronic stress as circumstances when animals are subjected to repeated episodes of acute

stress and the animals have time to recover between episodes and have a chance to learn and adapt to the stressful environment. In my procedure the animals are exposed to the stressor only once.

APPENDIX B

NOTE TO USERS

**Page(s) missing in number only; text follows.
Microfilmed as received.**

164

This reproduction is the best copy available.

UMI

APPENDIX B:

B.1 TIME COURSE DATA

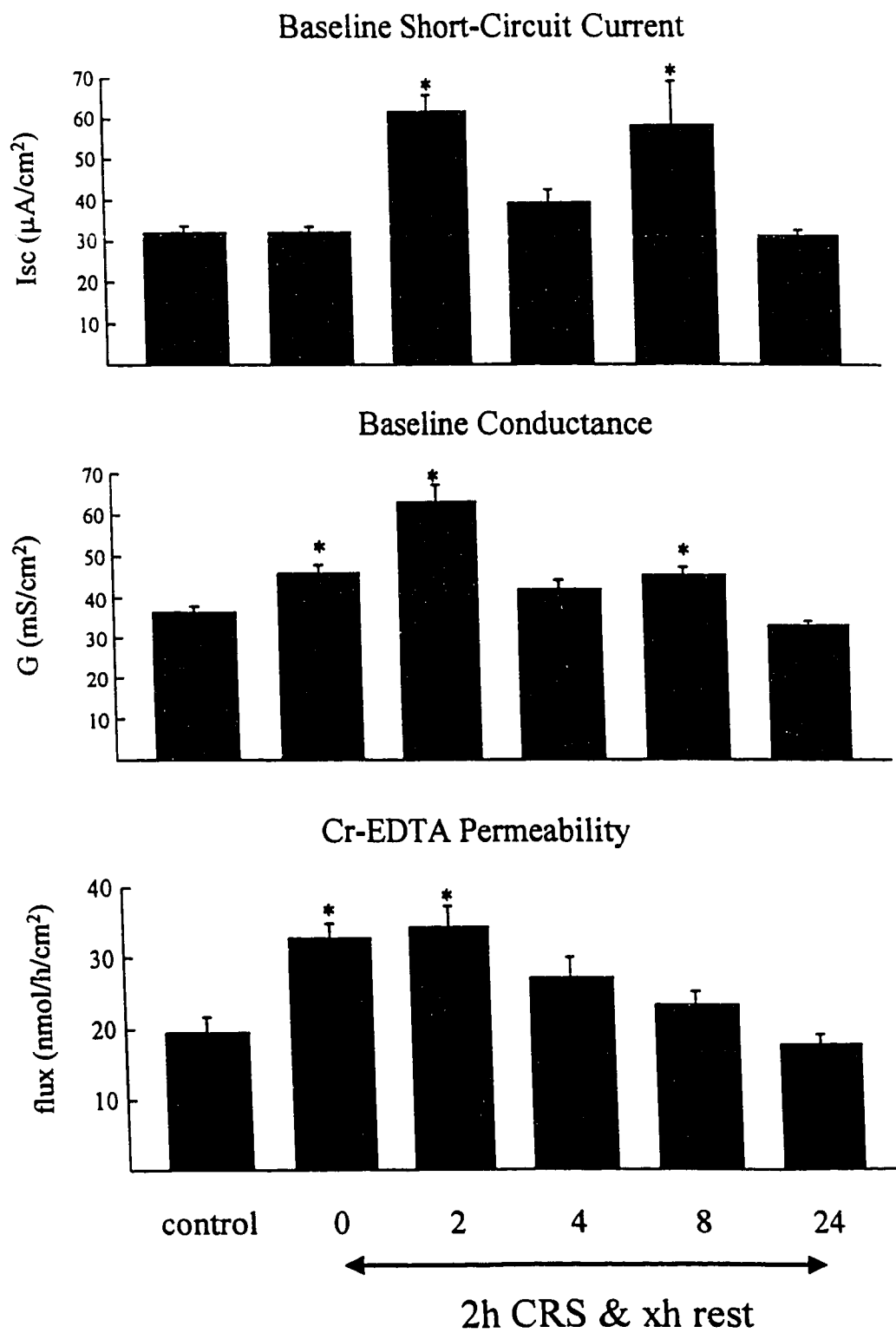
The purpose of this experiment was to reduce the time the experimental rats spent in a stressful environment and still produce measurable intestinal responses to stress in the Wistar Kyoto rats. To accomplish this we reduced the time rats spent in a stressful environment but included rest periods following the stress. We needed to know how long the rest periods needed to be to generate adequate responses to stress in the intestinal mucosa of the rats.

Wistar Kyoto rats were fasted overnight (approximately 14 hours) then at 8:00 am the rats were placed in the restraining tubes for 2 hours, which were located in a cold room at 4°C. Afterward, the rats' jejunum were removed immediately, when the rats were anaesthetized, or the rats were left for either 2, 4, 8 or 24 hours in their cage, at room temperature, before their jejunum were removed. The jejunum were stripped of their external muscle and the tissue mounted in Ussing chambers. Baseline short-circuit current, baseline conductance, and mucosal to serosal Cr-EDTA fluxes were measured and compared with jejunal tissue removed from fasted control rats.

All three measures showed the greatest response to stress at 2 hours rest following the 2 hours of cold restraint stress (see figure B.1). These values are nearly the same, for these three responses, as what we observed from jejunal tissue taken from rats subjected to 4 hours of cold restraint stress with the tissue removed immediately following the stress. There does seem to be a recurrence of a stress response at 8 hours post cold restraint stress; this may be a late phase reaction to the stress, more experiments with several more rest periods are required to verify this hypothesis. All responses are back to control levels within 24 hours of rest following the cold restraint stress. Therefore, the effects of acute stress on the intestinal tract appear to resolve themselves within a day of terminating the stress.

The greatest response to stress observed in the jejunal mucosa, from Wistar Kyoto rats, occurred following 2 hours of rest after the initial 2 hours of cold restraint stress.

Figure B.1 Time course of intestinal epithelial stress responses. Jejunal tissue from cold restraint stressed Wistar Kyoto rats was removed following different post-stress rest periods, at room temperature, and was analyzed and then compared to responses from tissues of control rats. The responses include baseline short-circuit current, baseline conductance, and mucosal to serosal fluxes of Cr-EDTA. The values are mean \pm SEM averaged for 6 rats, with 3-4 tissues/rat; * indicates $p < 0.05$, compared to control values, for the Neuman Keul's multiple comparison test.



NOTE TO USERS

**Page(s) missing in number only; text follows.
Microfilmed as received.**

168

This reproduction is the best copy available.

UMI

REFERENCES

- Adams, R.B., S.M. Planchon and J.K. Roche. 1993. IFN- γ modulation of epithelial barrier function. Time course, reversibility, and site of cytokine binding. *Journal of Immunology*, 150: 2356-2363.
- Almy T.P., F. Kern Jr., and M. Tulin. 1949. Alterations in colonic function in man under stress: experimental production of sigmoid spasm in healthy persons. *Gastroenterology*, 12:425-436.
- Andrianopoulos, G., C.T. Bombeck and L.M. Nyhus. 1986. Influence of maturation on activity-stress related pathology in the rat colon. *Physiology & Behaviour*, 36: 1105-1110.
- Atisook, K. And J.L. Madara. 1991. An oligopeptide permeates intestinal tight junctions at glucose-elicited dilatations. *Gastroenterology*, 100: 719-724.
- Barclay, G.R. and L.A. Turnberg. 1987. Effect of psychological stress on salt and water transport in the human jejunum. *Gastroenterology*, 93: 91-97.
- Barclay, G.R. and L.A. Turnberg. 1988a. Effect of cold-induced pain on salt and water transport in the human jejunum. *Gastroenterology*, 94: 994-998.
- Barclay, G.R. and L.A. Turnberg. 1988b. Effect of moderate exercise on salt and water transport in the human jejunum. *Gut*, 29: 816-820.
- Basso, N., A. Materia, A. Forlini and B.M. Jaffe. 1983. Prostaglandin generation in the gastric mucosa of rats with stress ulcer. *Surgery*, 94:104-108.
- Bhargava, K.P., M. Daas, G.P. Gupta and M.B. Gupta. 1980. Study of central neurotransmitters in stress-induced gastric ulceration in albino rats. *British Journal of Pharmacology*, 68: 765-772.
- Bijlsma P.B., A.J. Kiliaan, G. Scholten, M. Heyman, J.A. Groot, and J.A.J.M. Taminiau. 1996. Carbachol but not forskolin can increase mucosal to serosal transport of intact protein in rat ileum *in vitro*. *American Journal of Physiology*, 271:G147-G155.

- Bohmelt, A. H., G. Winneke and W. Boucsein. 1994. The effects of control on stress-induced gastric erosions: the use of active shuttle-box avoidance learning with yoked controls. *Physiology & Behaviour*, 55: 1101-1108.
- Bueno L., S.M. Collins, and J.L. Junien. 1989. *Stress and Digestive Motility*. John Libby Publishers, London, UK.
- Canil, C., I. Rosenshine, S. Ruschkowski, M.S. Donnenberg, J.B. Kaper and B.B. Finlay. 1993. Enteropathogenic *Escherichia coli* decreases the transepithelial electrical resistance of polarized epithelial monolayers. *Infection and Immunity*, 61: 2755-2762.
- Cannon, W.B.. 1929a. Bodily changes in pain, hunger, fear and rage: an account of recent researches into the function of emotional excitement. Appleton, New York, NY, USA.
- Cannon, W.B.. 1929b. The wisdom of the body. *Physiology Reviews*, 9:399-431.
- Carter E.A., R.G. Tompkins, E. Schiffrin, and J.F. Burke. 1990. Cutaneous thermal injury alters macromolecular permeability of rat small intestine. *Surgery*, 107:335-341.
- Castagliuolo I., J.T. LaMont, B. Qiu, S.M. Fleming, K.R. Bhaskar, S.T. Nikulasson, C. Kornetsky, and C. Pothoulakis. 1996. Acute stress causes mucin release from rat colon: role of corticotropin releasing factor and mast cells. *American Journal of Physiology*, 271: G884-G892.
- Cereijido, M. Evolution of ideas on the tight junction. 1991. In Tight Junctions. ed. M. Cereijido. CRC Press, Ann Arbor, MI. pg. 1-13.
- Chrousos, G.P. and P.W. Gold. 1995. Introduction. *Annals of the New York Academy of Sciences*, 771: xv-xviii.
- Cooke H.J. and R.A. Reddix. 1994. Neural regulation of intestinal electrolyte transport. In: Physiology of the Gastrointestinal Tract. 3rd edition, ed. LR Johnson. Raven Press, New York, NY, USA. 2083-2132.
- Crissinger, K.D. and D.N. Granger. 1995. Gastrointestinal blood flow. In: Textbook of Gastroenterology, ed. T. Yamada. J.B. Lippincott Co., Philadelphia, Pa., USA. pg. 518-545.
- Crofford, L.J., H. Sano, K. Karalis, E.A. Webster, T.C. Friedman, G.P. Chrousos and R.L. Wilder. 1995. Local expression of corticotropin releasing hormone in inflammatory arthritis. *Annals of the New York Academy of Sciences*, 771: 459-471.

- Diamond, J.M.. 1974. Tight and leaky junctions of epithelia: a perspective on kisses in the dark. *Federation Proceedings*, 33: 2220-2224.
- Dodd, J. And L.W. Role. 1991. The autonomic nervous system. In Principles of Neural Science, 3rd edition, eds. ER Kandel, JH Schwartz and TA Jessell. Elsevier, New York, NY, USA. pg. 761-775.
- Duchmann, R., I. Kaiser, E. Hermann, W. Mayet, K. Ewe and K-H. Meyer zum Buschenfelde. 1995. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease. *Clinical and Experimental Immunology*, 102: 448-455.
- Duffy L.C., M.A. Zielezny, J.R. Marshall, T.E. Byers, M.M. Weiser, J.F. Phillips, B.M. Calkins, P.L. Ogra and S. Graham. 1991. Relevance of major stress events as an indicator of disease activity prevalence in inflammatory bowel disease. *Behavioral Medicine*, 17:101-110, 1991.
- Dunn, A.J. and C.W. Berridge. 1990. Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses? *Brain Research Reviews*, 15: 71-100.
- Ekblad, E., C. Winther, R. Ekman, R. Hakanson and F. Sundler. 1987. Projections of peptide-containing neurons in rat small intestine. *Neuroscience*, 20: 169-188.
- Ekblad, E., R. Ekman, R. Hakanson and F. Sundler. 1988. Projections of peptide-containing neurons in rat colon. *Neuroscience*, 27: 655-674.
- Empey, L.R. and R.N. Fedorak. 1989. Effect of misoprostol in preventing stress-induced intestinal fluid secretion in rats. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 38: 43-8.
- Fasano, A., B. Baudry, D.W. Pumplun, S.S. Wasserman, B.D. Tall, J.M. Ketley and J.B. Kaper. 1991. *Vibrio cholerae* produces a second enterotoxin, which affects intestinal tight junctions. *Proceedings of the National Academy of Science, USA*. 88: 5242-5246.
- Frizzell, R.A. and S.G. Schultz. 1972. Ionic conductances of extracellular shunt pathway in rabbit ileum. Influence of shunt on transmural sodium transport and electrical potential differences. *The Journal of General Physiology*, 59: 318-346.
- Fromter, E.. 1972. The route of passive ion movement through the epithelium of *Necturus* gallbladder. *Journal of Membrane Biology*, 8: 259-301.

- Furness, J.B. and J.C. Bornstein. 1995. The enteric nervous system and its extrinsic connections. In Textbook of Gastroenterology, ed. T. Yamada. J.B. Lippincott Co., Philadelphia, Pa., USA. pg. 2-25.
- Furness, J.B. and M. Costa. 1987. The Enteric Nervous System. Churchill Livingstone, New York, N.Y., USA.
- Garrick, T.. 1990. The role of gastric contractility and brain thyrotropin-releasing hormone in cold restraint-induced gastric mucosal injury. *Annals of the New York Academy of Sciences*, 597: 51-70.
- Gaton, J., F. Fernandez de la Gandara and A. Velasco. 1993. The role of the neurotransmitters acetylcholine and noradrenaline in the pathogenesis of stress ulcers. *Comparative Biochemistry and Physiology*, 106C: 125-129.
- Glavin G.B.. 1980. Restraint ulcer: history, current research and future implications. *Brain Research Bulletin*, 5:51-58.
- Hanson, H.M. and D.A. Brodie. 1960. Use of the restrained rat technique for study of the antiulcer effect of drugs. *Journal of Applied Physiology*, 15: 291-294.
- Hecht, G., A. Koutsouris, C. Pothoulakis, J.T. LaMont and J.L. Madara. 1992. *Clostridium difficile* toxin B disrupts the barrier function of T₈₄ monolayers. *Gastroenterology*, 102: 416-423.
- Hilsden, R.J., J.B. Meddings and L.R. Sutherland. 1996. Intestinal permeability changes in response to acetylsalicylic acid in relatives of patients with Crohn's disease. *Gastroenterology*, 110: 1395-1403.
- Hollander, D., C.M. Vadheim, E. Brettholz, G.M. Petersen, T. Delahunty and J.I. Rotter. 1986. Increased intestinal permeability in patients with Crohn's disease and their relatives. *Annals of Internal Medicine*, 105: 883-885.
- Jacobson, K., P.C. Miceli, B.A. Vallance and S.M. Collins. 1997. The brain gut axis in IBD: stress reactivation of experimental colitis in rats requires concomitant luminal hapten challenge. *Gastroenterology*, 112: A1157.
- Johnson, E.O., T.C. Kamilaris, G.P. Chrousos and P.W. Gold. 1992. Mechanisms of stress: a dynamic overview of hormonal and behavioral homeostasis. *Neuroscience and Biobehavioral Reviews*, 16: 115-130.

- Kaunitz, J.D., K.E. Barrett and J.A. McRoberts. 1995. Electrolyte secretion and absorption: small intestine and colon. In Textbook of Gastroenterology, ed. T. Yamada. J.B. Lippincott Co., Philadelphia, Pa., USA. pg. 326-361.
- Kawahito, Y., H. Sano, S. Mukai, K. Asai, S. Kimura, Y. Yamamura, H. Kato, G.P. Chrousos, R.L. Wilder and M. Kondo. 1995. Corticotropin releasing hormone in colonic mucosa in patients with ulcerative colitis. *Gut*, 37: 544-551.
- Kimm, M.H., G.H. Curtis, J.A. Hardin, and D.G. Gall. 1994. Transport of bovine serum albumin across rat jejunum: role of the enteric nervous system. *American Journal of Physiology*, 266: G186-G193.
- Kitagawa, H., K. Kurahashi and M. Fujiwara. 1984. Water-immersion stress induces mucosal ischemia due to thromboxane A₂-like substance and ulcer formation in rats. *Japanese Journal of Pharmacology*, 35: 478-480.
- Kitajima, M., A. Shimizu, N. Sakai, S. Otsuka, M. Mogi, M. Nakajima, T. Kiuchi, Y. Ikeda and A. Oshima. 1991. Gastric microcirculation and its regulating factors in stress. *Journal of Clinical Gastroenterology*, 13 (supplement): S9-S17.
- Kopin, I.J. 1995. Definitions of stress and sympathetic neuronal responses. *Annals of the New York Academy of Sciences*, 771: 19-30.
- Lenz, H.J. 1989. Regulation of duodenal bicarbonate secretion during stress by corticotropin-releasing factor and β -endorphin. *Proceedings of the National Academy of Science, USA*, 86: 1417-1420.
- Lim, D.K., I.B. Hoskins, R.W. Rockhold and I.K. Ho. 1989. Comparative studies of muscarinic and dopamine receptors in three strains of rat. *European Journal of Pharmacology*, 165: 279-87.
- Madara, J.L., 1995. Epithelia: biological principles of organization. In Textbook of Gastroenterology, ed. T. Yamada. J.B. Lippincott Co., Philadelphia, Pa., USA. pg. 141-157.
- Madara, J.L. and J.S. Trier. 1994. The functional morphology of the mucosa of the small intestine. In: Physiology of the Gastrointestinal Tract. 3rd edition, ed. LR Johnson. Raven Press, New York, NY, USA. 1577-1622.
- Maneker, M.S. 1976. Effect of autonomic drugs on the stress-induced acute gastric ulceration in rat. *Indian Journal of Physiology and Pharmacology*, 20: 160-63.

- Marcial, M.A., S.L. Carlson and J.L. Madara. 1984. Partitioning of paracellular conductance along the ileal crypt-villus axis: a hypothesis based on structural analysis with detailed consideration of tight junction structure-function relationships. *Journal of Membrane Biology*, 80: 59-70.
- Mason, J.W. 1968. "Over-all" hormonal balance as a key to endocrine organization. *Psychosomatic Medicine*, 30: 791-808.
- Mason, J.W. 1975. Emotion as reflected in patterns of endocrine integrations. In Emotions - Their Parameters and Measurements, ed. L. Levi. Raven. New York, NY, USA. pg. 143-181.
- Mason, W.A. 1978. Social experience and primate cognitive development. In: The Development of Behavior: Comparative and Evolutionary Aspects, eds. G.M. Burghardt and M. Bekoff. Garland. New York, NY, USA. pg. 233-251.
- May G.R., L.R. Sutherland and J.B. Meddings. 1993. Is small intestinal permeability really increased in relatives of patients with Crohn's disease? *Gastroenterology*, 104:1627-1632.
- McRae S., K. Younger, D.G. Thompson and D.L. Wingate. 1982. Sustained mental stress alters human jejunal motor activity. *Gut*, 23:404-409.
- Meddings, J.B., I.R. Gibbons and M. Swain. 1997. Stress induced alterations in gastrointestinal permeability. *Gastroenterology*, 112: A385.
- Moberg, G.P. 1985. Biological response to stress: key to assessment of animal well-being. In: Animal Stress, ed. G Moberg. Williams & Wilkins Co., Baltimore, Md., USA. pg. 27-49.
- Monnikes, H., B.G. Schmidt, H.E. Raybould and Y. Taché. 1992. CRF in the paraventricular nucleus mediates gastric and colonic motor response to restraint stress. *American Journal of Physiology*, 262: G137-G143.
- Munkholm, P., E. Langholz, D. Hollander, K. Thornberg, M. Orholm, K.D. Katz and V. Binder. 1994. Intestinal permeability in patients with Crohn's disease and ulcerative colitis and their first degree relatives. *Gut*, 35: 68-72.
- Nosalova, V., I. Juranek and A. Babulova. 1991. Effect of pentacaine and ranitidine on gastric mucus changes induced by cold-restraint stress in rats. *Agents and Actions*, 33: 164-166.

- Orlando, R.C., D.E. Hernandez, A.J. Prange Jr. and C.B. Nemeroff. 1985. Role of the autonomic nervous system in the cytoprotective effect of neurotensin against gastric stress ulcers in rats. *Psychoneuroendocrinology*, 10: 149-157.
- Overmier, J.B. 1988. Psychological determinants of when stressors stress. In: Neurobiological Approaches to Human Disease, eds. D Hellhammer, I Florin and H Weiner. Hans Huber Publishers, Toronto, Ont., Can.. pg 237-259.
- Pare, W.P.. 1989a. Strain, Age, but not gender influence ulcer severity induced by water-restraint stress. *Physiology & Behaviour*, 45: 627-632.
- Pare, W.P.. 1989b. Stress ulcer susceptibility and depression in Wistar Kyoto (WKY) rats. *Physiology & Behaviour*, 46: 993-998.
- Phillips T.E., T.L. Phillips and M.R. Neutra. 1987. Macromolecules can pass through occluding junctions of rat ileal epithelium during cholinergic stimulation. *Cell and Tissue Research*, 247: 547-554.
- Qiu, B.S., B.A. Vallance, P.A. Blennerhassett and S.M. Collins. 1997. Convergence of stress and intestinal luminal exposure to hapten are required for the reactivation of colitis in the mouse. *Gastroenterology*, 112: A1065.
- Ray, A., R.M. Sullivan and P.G. Henke. 1987. Adrenergic modulation of gastric stress pathology in rats: a cholinergic link. *Journal of the Autonomic Nervous System*, 20: 265-268.
- Redei, E., W.P. Pare, F. Aird and J. Kluczynski. 1994. Strain differences in hypothalamic-pituitary adrenal activity and stress ulcer. *American Journal of Physiology*, 266: R353-R360.
- Rhodes R.S. and M.J. Karnovsky. 1971. Loss of macromolecular barrier function associated with surgical trauma to the intestine. *Laboratory Investigations*, 25: 220-229.
- Role, L.W. and J.P. Kelly. 1991. The brain stem: cranial nerve nuclei and the monoaminergic systems. In Principles of Neural Science, 3rd edition, eds. ER Kandel, JH Schwartz and TA Jessell. Elsevier, New York, NY, USA. pg. 683-699.
- Sadowski, D.C. and J.B. Meddings. 1993. Luminal nutrients alter tight-junction permeability in the rat jejunum: an *in vivo* perfusion model. *Canadian Journal of Physiology and Pharmacology*, 71: 835-839.

- Sanderson, I.R. and W.A. Walker. 1993. Uptake and transport of macromolecules by the intestine: possible role in clinical disorders (an update). *Gastroenterology*, 104: 622-639.
- Santos, J., E. Saperas, C. Nogueiras, M. Mourelle, M. Antolin, A. Cadahia and J-R. Malagelada. 1998. Release of mast cell mediators into the jejunum by cold pain stress in humans. *Gastroenterology*, 114: 640-648.
- Sartor, R.B. 1995. Current concepts of the etiology and pathogenesis of ulcerative colitis and Crohn's disease. *Gastroenterology Clinics of North America*, 24: 475-507.
- Selye, H. 1936. A syndrome produced by diverse nocuous agents. *Nature*, 138: 32.
- Selye, H. 1946. The general adaptation syndrome and the diseases of adaptation. *The Journal of Clinical Endocrinology*, 6: 117-230.
- Selye, H. 1950. *Stress*. Acta. Montreal, Que., Can..
- Selye, H. 1975. *The Stress of Life*. McGraw Hill. New York, NY, USA.
- Senay, E.C. and R.J. Levine. 1967. Synergism between cold and restraint for rapid production of stress ulcers in rats. *Proceedings of the Society for Experimental Biology and Medicine*, 124: 1221-1223.
- Sengupta, J.N. and G.F. Gebhart. 1994. Gastrointestinal afferent fibres and sensation. In: Physiology of the Gastrointestinal Tract. 3rd edition, ed. LR Johnson. Raven Press, New York, NY, USA. 483-519.
- Shanahan, F. 1994. The intestinal immune system. In: Physiology of the Gastrointestinal Tract. 3rd edition, ed. LR Johnson. Raven Press, New York, NY, USA. pg. 643-684.
- Shichijo, K., M. Ito and I. Sekine. 1991. The mechanism of low susceptibility to stress in gastric lesions of spontaneously hypertensive rats. *Life Sciences*, 49: 2023-2029.
- Sines, J.O.. 1959. Selective breeding for the development of stomach lesions following stress in the rat. *Journal of Comparative Physiological Psychology*, 52: 615-617.
- Stratakis, C.A. and G.P. Chrousos. 1995. Neuroendocrinology and pathophysiology of the stress system. *Annals of the New York Academy of Sciences*, 771: 1-18.
- Takeda, H., M. Asaka, K. Matsuno, T. Ohtaki and T. Miyazaki. 1992. Stress-induced gastric mucosal lesion and platelet aggregation in rats. *Journal of Clinical Gastroenterology*, 14 (supplement): S145-S148.

- Tromm A., C.D. Tromm, D. Huppe, U. Schwegler, M. Krieg, and B. May. 1992. Evaluation of different laboratory tests and activity indices reflecting the inflammatory activity of Crohn's disease. *Scandinavian Journal of Gastroenterology*, 27: 774-778.
- van Tol, E.A.F., P. Petrusz, P.K. Lund, M. Yamauchi and R.B. Sartor. 1996. Local production of corticotropin releasing hormone is increased in experimental intestinal inflammation in rats. *Gut*, 39: 385-392.
- Wang, J-Y. And L.R. Johnson. 1989. Induction of gastric and duodenal mucosal ornithine decarboxylase during stress. *American Journal of Physiology*, 257: G259-G265.
- Williams, C.L., R.G. Villar, J.M. Peterson and T.F. Burks. 1988. Stress-induced changes in intestinal transit in the rat: a model for irritable bowel syndrome. *Gastroenterology*, 94: 611-621.
- Wittman T., F. Crenner, F. Angel, L. Hanusz, C. Ringwald and J.F. Grenier. 1990. Long duration stress. Immediate and late effects on small and large bowel motility in rat. *Digestive Diseases and Science*, 35: 495-500.
- Wood, J.D. 1995. The brain-gut axis. In Textbook of Gastroenterology, ed. T. Yamada. J.B. Lippincott Co., Philadelphia, Pa., USA. pg. 71-86.
- Wood, J.D., O.C. Peck, H.S. Sharma, J.I. Hernandez, J.V. Rodriguez, M.A. Rodriguez, M.J. Stonerook, K.S. Tefend, J. Gardezabal, F. Nassar, S. Lhotak. 1996. Evidence that environmental stress initiates and the fecal microflora sustains the inflammatory state found in the cotton-top tamarin model for ulcerative colitis. *Gastroenterology*, 110: A1047.
- Yacyshyn, B.R. and J.B. Meddings. 1995. CD45RO expression on circulating CD19⁺ B cells in Crohn's disease correlates with intestinal permeability. *Gastroenterology*, 108: 132-137.
- Yano, S., M. Akahane and M. Harada. 1978. Role of gastric motility in development of stress-induced gastric lesions of rats. *Japanese Journal of Pharmacology*, 28: 607-615.