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Molecular Interactions between *Entamoeba histolytica* and Colonic Mucins

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Abstract

The enteric protozoan parasite *Entamoeba histolytica* is the etiologic agent of the disease amebiasis which is characterized by colitis or hepatic lesions. Amebae colonize the colon by binding to mucous glycoproteins (mucins). Secretory mucins provide the gel nature to mucus and are a vital component of epithelial barrier function. Mucins prevent contact-dependent cytolysis of colonic cells by *E. histolytica*. To possibly circumvent this barrier, the parasite secretes a potent yet unidentified mucin secretagogue, which could deplete the stored mucin pool and render the mucous layer less protective. The objective of this study was to investigate the molecular mechanisms by which *E. histolytica* modulates colonic mucin exocytosis. We showed that *E. histolytica* converts exogenous arachidonic acid to prostaglandin E_2 (PGE_2), a known mucin secretagogue and potential mechanism by which the parasite evokes mucin secretion. Conversion was via a novel cyclooxygenase-like activity and was inhibitable with the known cyclooxygenase inhibitor aspirin. To study *E. histolytica*-mucin interactions, we developed an *in vitro* model of LS174T human colonic epithelial cells that secrete mucin constitutively and in response to mucin agonists. Highly purified mucins isolated from LS174T cells markedly inhibited amebic adherence to target cells and the mucous barrier protected the LS174T monolayers from amebic cytolysis. We have identified that Gal and GalNAc residues (O-linked sugars) of mucins are the protective moiety as O- but not N-linked glycosylation inhibitors decreased their protective effect. To understand how mucins are regulated during intestinal amebiasis and in the inflamed gut, we determined that PGE_2 binds the EP_4 receptor on LS174T cells and in rat colon to stimulate cyclic adenosine monophosphate-dependent mucin exocytosis. Taken together, these studies delineate how *E. histolytica* modulates host responses during infection to allow the parasite to survive and persist in the host.

Abrégé

Le parasite entérique protozoaire *Entamoeba histolytica* est l'agent étiologique de l'amibiase, une maladie caractérisée par des colites et des lésions hépatiques. Les amibes colonisent le côlon en s'accrochant aux glycoprotéines du mucus, les mucines. Les mucines sécrétées confèrent la nature de gel au mucus et sont des composantes essentielles de la barrière épithéliale. Les mucines empêchent la cytolyse des cellules du côlon due au contact avec *E. histolytica*. Pour contourner cette barrière, il est possible que le parasite sécrète une sécrétagogue puissante et inconnue qui viderait les stocks cellulaires de mucines et rendrait la couche de mucus moins protectrice. L'objectif de cette étude était d'identifier le mécanisme moléculaire utilisé par *E. histolytica* pour moduler l'exocytose des mucines du côlon. Nous avons démontré que *E. histolytica* convertit l'acide arachidonique exogène en prostaglandine E_2 (PGE_2), une sécrétagogue de mucines connue, ce qui pourrait être un mécanisme par lequel le parasite stimule la sécrétion de mucines. Cette conversion se fait à l'aide d'une activité semblable à la cyclooxygénase, qui peut être bloquée par l'aspirine, un inhibiteur de cyclooxygénase bien connu. Pour pouvoir étudier les interactions entre *E. histolytica* et les mucines, nous avons développé un modèle *in vitro* utilisant la lignée de cellules épithéliales humaines du côlon LS174T; ces cellules sécrètent des mucines de façon constitutive et en réponse aux agonistes de sécrétion de mucines. Les mucines purifiées provenant des cellules LS174T ont inhibé de façon marquée l'adhérence des amibes aux cellules cibles, et la couche de mucus sur les cultures de cellules LS174T les a protégé contre la cytolyse due aux amibes. Nous avons trouvé que les résidus Gal et GalNAc (O-monosaccharides) des mucines sont ceux qui confèrent cette protection, puisque les inhibiteurs de O-glycosylation, mais non ceux de N-glycosylation ont diminué l'effet protecteur. En essayant de comprendre comment la production de mucines est régulée

durant l'amibiase intestinale et dans l'intestin enflammé, nous avons déterminé que la PGE_2 se lie au récepteur EP_4 sur les cellules LS174T et dans le côlon des rats, et stimule une exocytose de mucines qui est dépendante de l'adénosine monophosphorique cyclique. En somme, ces études clarifient comment *E. histolytica* module la réponse de son hôte durant l'infection pour permettre au parasite de survivre dans l'hôte et d'y persister.

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Finally, I would like to thank my wife, Kimberly, for her constant support and encouragement and my daughter, Victoria, for the inspiration. I would also like thank my parents, Sharon and Jacques, my brother Paul and my in-laws, George and Patricia, for their encouragement and support.

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List of Abbreviations

AA	arachidonic acid
ALA	amebic liver abscess
ASA	aspirin
$[Ca^{2+}]_i$	intracellular calcium
$[cAMP]_i$	intracellular cyclic adenosine monophosphate
cDNA	complementary DNA
C/EBP	CCAAT/enhancer-binding protein
CHO	Chinese hamster ovary
COX	cyclooxygenase
CRE	$[cAMP]_i$ responsive element
DSS	dextran sodium sulfate
EhNP	*E. histolytica* nuclear protein
EIA	enzyme immunoassay
Gal	galactose
GalNAc	N-acetylgalactosamine
GC/MS	gas chromatography/mass spectometry
GlcNAc	N-acetylglucosamine
GTP	guanosine triphosphate
HPLC	high performance liquid chromatography
IBMX	isobutylmethylxanthine
IFN	interferon
IL	interleukin

INDO	indomethacin
IR	irregular repeat
kb	kilobase
kD	kilodalton
LDCV	large dense core vesicles
LPS	lipopolysaccharide
mRNA	messenger RNA
NSAID	non-steroidal antiinflammatory drug
NF-κB	nuclear factor-κB
PBMC	peripheral blood mononuclear cells
PG	prostaglandin
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear phagocytes
TNF	tumor necrosis factor
TRE	tumor responsive element
VNTR	variable number of tandem repeats
vWF	von Willibrand factor

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Additional material (procedural and design data, as well as descriptions of the equipment used) must be provided where appropriate and in sufficient detail (*e.g.* in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent;** supervisors must attest to the accuracy of such claims at the Ph.D. Oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.

Statement of Originality

The following aspects described in this thesis are considered contributions of original knowledge:

Manuscript I

E. histolytica releases an unidentified mucin secretagogue and immunosuppressive substances. This is the first demonstration that *E. histolytica* produces the immunomodulating agent and mucin secretagogue, prostaglandin E_2 (PGE_2). Moreover, it is the first study to characterize the cyclooxygenase (COX) activity within the parasite. Characterization of the putative amebic COX enzyme revealed a molecular mass, isoelectric point and pharmacologic profile that differed from mammalian COX-1.

Manuscript II

This is the first description of an i*n vitro* model using colonic cells to study *E. histolytica*-mucin interactions. We demonstrated that mucin O-linked sugars are the protective moiety of the mucin molecule and prevent amebic adherence and killing of colonic epithelial cells (LS174T) by *E. histolytica*.

Manuscript III

This is the first report describing in detail the receptor-coupled signaling pathway involved in PGE_2-mediated mucin exocytosis. We demonstrated using various pharmacologic agonists that PGE_2 signals via the EP_4 receptor to stimulate cyclic adenosine

monophosphate-dependent mucin secretion in the colonic cell line LS174T and in rat colon. These findings further our understanding of how mucin secretion is regulated during inflammation and intestinal infection with *E. histolytica*.

Statement of Authorship

This thesis consists of three manuscripts co-authored with my supervisor, Dr. Kris Chadee. Dr. Chadee provided financial resources for the laboratory work, advice on experimental design and corrections of the manuscripts and thesis. I was responsible for experimental design, laboratory work, data analysis and preparation of the manuscripts. Manuscript II was co-authored by Kathy Keller and Janet Grove, both of whom provided expert technical assistance.

Section I: Literature Review

Introduction

The protozoan parasite *Entamoeba histolytica* is the etiologic agent of the disease amebiasis. The parasite colonizes the mucous layer of the colon and causes tissue pathology such as fluid secretion, dysentery, focal erosions, ulceration and colitis. For reasons unknown, the organism can invade the intestinal epithelium and migrate to soft organs, usually the liver, to form abscesses.

Complex cellular interactions occur between trophozoites and host cells during the pathogenesis of invasive amebiasis. Amebic trophozoites release substances that modulate a variety of host cellular functions which include intestinal ion balance, mucous secretion, attraction of neutrophils and suppression of immune cell responses. Within the colon, ameba colonize and adhere to mucous glycoproteins known as mucins (1). Mucins are gel forming molecules secreted by goblet cells that protect the colon from luminal irritants and physical stresses (2). The parasite expresses a lectin on its surface known as the galactose inhibitable adherence lectin which it uses to bind with high affinity to mucins (1, 3). *E. histolytica* trophozoites stimulate mucin secretion by releasing an unidentified secretagogue (4). It is unknown how the parasite overcomes the protective mucous layer but it was suggested that prolonged exposure to the secretagogue may deplete the stored mucin pool (4). Furthermore, washout of luminal contents during bouts of diarrhea may expose the epithelium. In animal models of infection, mucus depletion preceded epithelial damage (5, 6). Recently, our laboratory demonstrated that amebic secretory components and purified prostaglandin E_2 (PGE_2) stimulated production of the chemokine interleukin 8 (IL-8) by the human colonic epithelial cell line T84 (7, 8). IL-8 is a potent chemoattractant and activating factor for neutrophils (9). In intestinal amebiasis, recruitment of neutrophils to the lamina propria and mucosal layer occurs early and may initiate or exacerbate tissue

injury through release of proteases and reactive oxygen intermediates, a process which may facilitate invasion by the parasite (5, 10, 11).

PGE_2 is a lipid product of the arachidonic acid pathway produced by the enzymes cyclooxygenase (COX)-1 or COX-2. Inflammatory states are characterized by increased production of PGE_2 derived from the inducible COX-2 enzyme. PGE_2 is considered a proinflammatory agent: it causes vasodilation, enhances histamine- and bradykinin-induced edema and stimulates pain and fever (12). Moreover, it is a potent immunomodulating agent that suppresses numerous macrophage, T cell and B cell functions (13). Within the liver, *E. histolytica* encounters a number of host defense cells which are suppressed and cannot eliminate the parasite. Increased production of PGE_2 by amebic liver abscess macrophages and hepatic cells was reported and treatment of infected gerbils with the COX inhibitor indomethacin reduced abscess size (14, 15). Although COX enzymes have only been described in vertebrates, several parasites were shown to produce prostaglandins. It is unknown whether *E. histolytica* contains COX activity and produces prostaglandins. Production of PGE_2 by trophozoites may explain some of the pathophysiology and immunomodulation that occurs during infection. **The objective of this study was to determine the mechanisms by which *E. histolytica* modulates mucins in the colon.** The specific aims were:

1). ***To characterize PGE_2 production by E. histolytica;***

2). ***To develop an in vitro model to study E. histolytica-mucin interactions;***

3). ***To characterize the receptor-coupled signaling involved in PGE_2-mediated mucin exocytosis.***

These studies have identified a parasite-derived molecule (PGE_2) that may play a vital role

in pathogenesis and immune evasion. Moreover, the development of an *in vitro* model to study parasite-mucin interactions is crucial to our understanding of how mucins are regulated during inflammatory states.

References

1. Chadee K, Petri Jr. WA, Innes DJ, Ravdin JI. Rat and human colonic mucins bind to and inhibit the adherence lectin of *Entamoeba histolytica.* J Clin Invest 1987;80:1245-1254.

2. Forstner JF, Forstner GG. Gastrointestinal mucus. In Johnson LR, ed. Physiology of the gastrointestinal tract. Volume 2, 3rd ed. New York: Raven, 1995:1255-1283.

3. Petri Jr. WA, Smith RD, Schlesinger PH, Murphy CF, Ravdin JI. Isolation of the galactose-binding lectin that mediates in vitro adherence of *Entamoeba histolytica.* J Clin Invest 1987;80:1238-1244.

4. Chadee K, Keller K, Forstner J, Innes DJ, Ravdin JI. Mucin and nonmucin secretagogue activity of *Entamoeba histolytica* and cholera toxin in rat colon. Gastroenterology 1991;100:986-997.

5. Chadee K, Meerovitch E. *Entamoeba histolytica*: early progressive pathology in the cecum of the gerbil (*Meriones unguiculatus*). Am J Trop Med Hyg 1985;34:283-291.

6. Chadee K, Meerovitch E. The Mongolian gerbil (*Meriones unguiculatus*) as an experimental host for *Entamoeba histolytica.* Am J Trop Med Hyg 1984;33:47-54.

7. Yu Y, Chadee K. *Entamoeba histolytica* stimulates interleukin-8 from human colonic epithelial cell without parasite-enterocyte contact. Gastroenterology 1997;112:1536-1547.

8. Yu Y, Chadee K. Prostaglandin E_2 stimulates IL-8 gene expression in human colonic epithelial cells by a posttranscriptional mechanism. J Immunol 1988;161:3746-3752.

9. Baggiolini M, Dewbald B, Moser B. Interleukin-8 and related chemotactic cytokines--CXC and CC chemokines. Adv Immunol 1994;55:97-197.

10. Prathap K, Gilman R. The histopathology of acute intestinal amebiasis. A rectal biopsy study. Am J Pathol 1970;60:229-246.

11. Martinez-Palomo A, Tsutsumi V, Anaya-Velazquez F, Gonzalez-Robles A. Ultrastructure of experimental intestinal invasive amebiasis. Am J Trop Med Hyg 1989;41:273-279.

12. Williams KI, Higgs GA. Eicosanoids and inflammation. J Pathol 1988;156:101-110.

13. Phipps RP, Stein SH, Roper RL. A new view of prostaglandin E regulation of the immune response. Immunology Today 1991;12:349-351.

14. Wang W, Chadee K. *Entamoeba histolytica* alters arachidonic acid metabolism in macrophages in vitro and in vivo. Immunology 1992;76:242-250.

15. Sanchez-Ramirez B, Escalante B, Rosales-Encina JL, Talamas-Rohana P. Role of

prostaglandin E_2 in amebic liver abscess formation in hamsters. Prostaglandins 1997;53:411-421.

Chapter 1. Host/parasite interactions in amebiasis

1.1 *E. histolytica* life cycle

The human pathogen *E. histolytica* is the etiologic agent of the disease amebiasis. Infection occurs when cysts residing outside the host come into contact with food or water that is ingested. The average size of a cyst is 12 μm and can contain one to four nuclei (1). Trophozoites contain a single nuclei and are larger with an average diameter of 25 μm (1, 2). Excystation occurs in the small intestine and is mediated *in vitro* by a low oxygen tension, the presence of inorganic salts and optimal osmotic conditions (3). Inducing factors of excystation *in vivo* are not fully understood. Trophozoites migrate to and colonize the large intestine. Environmental stress causes trophozoites to encyst in the colon. *E. histolytica* colonizes the large intestine and excreted with stool in cyst or trophozoite form, completing the life cycle.

1.2 *E. histolytica* infections

Invasive amebiasis is characterized by acute or chronic colitis with dysentery, or liver abscess. Symptoms of intestinal disease include lower abdominal pain with presence of bloody diarrhea (4). However, in most cases, the parasite causes asymptomatic intestinal infection. Trophozoites colonize the mucous layer of the large intestine and for reasons unknown, invade the colonic epithelium. Invasion of the colonic mucosa occurs when amebae overcome the intestinal mucous blanket and adhere to mucosal epithelial cells whereby these cells are lysed by a contact dependent mechanism (5). Ulceration and penetration into tissues proceed, culminating in liver abscess. Amebic liver abscesses (ALA) are the most common extraintestinal infections. Symptoms include right-upper-quadrant pain and fever (6). Approximately 50 million cases of invasive disease occur each

year leading to 50,000-100,000 deaths (7).

1.3 Adherence and cytolysis

Trophozoites that colonized the mucous layer of the colon are swept out into the lumen by peristalsis and excreted in faeces. Mucins mimic surface glycoproteins of cells lining the gastrointestinal tract and act as receptors for *E.histolytica* and other intestinal pathogens (8). Rat and human colonic mucins have been shown to inhibit *E. histolytica* adherence to a number of mammalian host cells *in vitro* (9, 10). Trophozoites bind with high affinity to Gal and GalNAc residues on mucins via its Gal/GalNAc inhibitable adherence lectin (Gal lectin) and prevents attachment of *E. histolytica* to the colonic epithelium. However, trophozoites can overcome the mucous blanket by an unknown mechanism, adhere to the colonic epithelium and initiate the disease process. Adherence to epithelial cells is mediated by the Gal lectin (11). The 170 kilodalton (kD) heavy subunit of the lectin is involved in direct binding and is anchored to the amebic cytoplasmic membrane by a 35 kD light subunit. Amebic adherence via the Gal lectin was required for cytolysis of host cells: Gal or GalNAc monomers inhibited lysis of Chinese hamster ovary (CHO) cells *in vitro* (12-14). Purified rat and human colonic mucins were shown to be 10,000 times more effective by weight than the Gal/GalNAc monomers at inhibiting amebic adherence (10).

E. histolytica releases several molecules that contribute to the cytolytic mechanism of the parasite. Trophozoites release cysteine proteinases which may contribute to tissue destruction by degrading extracellular matrix (reviewed in 15). They also contain phospholipase A and a pore-forming molecule known as amebapore, which may contribute to the disruption of cellular membranes (16, 17).

1.4 Modulation of host responses in intestinal disease

E. histolytica intestinal infections are characterized by dysentery, ulcers and colitis. Crude amebic lysates induced Cl^- secretion in rat colon by stimulating $[cAMP]_i$ via increased PGE_2 production and inhibited absorption of Na^+ (18, 19). Interference with electolyte balances most likely contributes to the diarrhea observed in intestinal amebiasis.

Infiltration of neutrophils in the lamina propria and mucosal layer occurs during intestinal infections with *E. histolytica* (20, 21). Amebic secretory components induced IL-8 production by the human colonic enterocytic cell line T84 (22). IL-8 is a potent chemotactic and activating factor for neutrophils (23). The recruitment of neutrophils into the mucosa may initiate inflammation, as is seen in other inflammatory bowel diseases (24). Release of hydrolytic enzymes due to neutrophil lysis by trophozoites may contribute to intestinal tissue pathology. The destruction of colonic epithelial cells may facilitate amebic invasion and further tissue damage. It was hypothesized that mucus hypersecretion may deplete mucin stores and with subsequent washout with diarrheal excretions, expose the colonic epithelium (25). In animal models of infection, invasion of the colonic mucosa by *E. histolytica* is preceded by depletion of colonic mucins (21, 26). Trophozoites were shown to release a potent mucin secretagogue activity in rat colonic loop studies (25).

1.5 Cellular responses during progression of hepatic disease

A systemic immune response develops during invasive disease. Antibodies against amebic antigens, predominantly the serine-rich *E. histolytica* protein and the Gal lectin, were detected in human serum (27-29). Trophozoites have the innate ability to "cap" and shed antibodies that have adhered to its plasma membrane (30). Moreover, trophozoites are resistant to complement-mediated lysis (31, 32). Amebae enter the liver via the hepatic

portal circulation and become trapped in the portal radicles and liver sinusoids (33, 34). In hamster and gerbil liver, infiltration of polymorphonuclear leukocytes (PMNs) occurred rapidly, within 60 minutes and were the prominent cell type found surrounding trophozoites during early infection (35, 36). However, these cells were unable to phagocytize amebae and were consequently lysed by the trophozoites. The release of hydrolytic enzymes by lysed PMNs may have contributed to the destruction of surrounding hepatocytes (37). As the infection progressed, macrophages and histiocytes surrounded and penetrated the fibrous wall of the lesion but were unable to kill trophozoites (36). Histiocytes were also lysed by the trophozoites. Coalescence of several granulomas resulted in the formation of a cavitary abscess in the gerbil liver and was thought to parallel the development of the ALA in humans (36).

It was suggested that T cells control the growth of abscesses; T cell depletion was observed in lymph nodes and spleens of infected gerbils and were found surrounding the abscess (36). Moreover, thymectomy or immunization with anti-T cell serum of hamsters exacerbated hepatic disease (37). Antigen-activated $CD8^+$ T cells and T cells from ALA patients were cytotoxic to trophozoites *in vitro* (38, 39). Cytokines produced by $CD4^+$ T cells may activate effector cells for amebicidal activity. The Th1 cytokine IFN-γ activated macrophages, peripheral blood mononuclear cells (PBMC) and neutrophils to kill amebae *in vitro* (40-43). Killing of amebic trophozoites by macrophages and neutrophils was mediated by H_2O_2 (41, 43). Activated murine macrophages were shown to kill amebae through production of NO (44). It was suggested that a Th1-type cytokine response, characterized by production of IFN-γ, IL-2 and TNF which drives cell-mediated immunity, may be protective against amebic infections (45). Gerbil cytokine profiles indicated a Th0-type response (IL-2 and IL-4) with strong lymphoproliferative responses during early (day 5) abscess development (45). Proliferation and cytokine production were suppressed

during acute disease (day 20) whereas increased IL-2 production (and low IL-4) and lymphoproliferation occurred between days 30 to 60, during which abscess size decreased.

Suppression of macrophage and T cell functions occurs during invasive disease. Macrophages in the vicinity of hepatic lesions are suppressed, whereas, those distal to the abscess are in an activated state (46). TNF-α expression by naive macrophages was inhibited by pretreatment with amebic products prior to stimulation with LPS- or IFN-γ and LPS (47). Macrophage production of IL-1 was inhibited and ALA macrophages did not produce H_2O_2 when stimulated with PMA *in vitro* (46). Moreover, IFN-γ-induced expression of macrophage class II major histocompatibility complex Ia molecule was inhibited by pretreatment with amebic secretory products or amebic proteins (48). This could dramatically affect T cell activation and proliferation in response to antigen which in turn could result in low production of activating cytokines for macrophages. Indeed, antigen-specific proliferation was reduced in invasive amebiasis (49, 50). Moreover, T cells from patients with ALA showed 93% reduction in IFN-γ production in the presence of patient serum as compared to control serum (51). High production of immunosuppressive PGE_2 from ALA macrophages was reported and treatment with indomethacin partially reversed the inhibitory effect of amebae on Ia expression (48, 52). Production of PGE_2 by host cells may be key in suppressing immune defenses as indomethacin treatment of hamsters with amebic liver abscess decreased the elevated plasma PGE_2 levels to control levels and reduced liver abscess weight by 30% (53).

1.6 References

1. Smith JW, McQuay RM, Ash LR, Melvin DM, Orihel TC, Thompson JH. Diagnostic medical parasitology: intestinal protozoa. 1979.

2. Noble ER, Noble GA. Parasitology: the biology of animal parasites. 4th ed. Philadelphia: Lea and Febiger, 1976.

3. Chayen A, Avron B, Nuchamowitz Y, Mirelman D. Appearance of sialoglycoproteins in encysting cells of *Entamoeba histolytica*. Infect Immun 1988;56:673-681.

4. Petri Jr. WA. Recent advances in amebiasis. Crit Rev Clin Lab Sci 1996;33:1-37.

5. Ravdin JI. Pathogenesis of disease caused by *Entamoeba histolytica*: studies of adherence, secreted toxins, and contact-dependent cytolysis. Rev Infect Dis 1986;8:247-260.

6. Reed SL. Amebiasis: an update. Clin Infect Dis 1992;14:385-393.

7. Walsh JA. Problems in recognition and diagnosis: estimation of the global magnitude of morbidity and mortality. Rev Infect Dis 1986;8:228-238.

8. Tse S-K, Chadee K. The interaction between intestinal mucus glycoproteins and enteric infections. Parasitol Today 1991;7:163-172.

9. Chadee K, Johnson ML, Orozco E, Petri Jr. WA, Ravdin JI. 1988. Binding and internalization of rat colonic mucins by the galactose/N-acetyl-D-galactosamine adherence lectin of *Entamoeba histolytica. J. Infect. Dis*. 1988;158:398-406.

10. Chadee K, Petri Jr. WA, Innes DJ, Ravdin JI. Rat and human colonic mucins bind to and inhibit adherence lectin of *Entamoeba histolytica*. J Clin Invest 1987;80:1245-1254.

11. Petri Jr. WA, Smith RD, Schlesinger PH, Murphy CF, Ravdin JI. Isolation of the galactose-binding lectin that mediates *in vitro* adherence of *Entamoeba histolytica.* J Clin Invest 1987;80:1238-1244.

12. Ravdin JI, Guerrant RL. Role of adherence in cytopathogenic mechanisms of *Entamoeba histolytica*: study with mammalian tissue culture cells and human erythrocytes. J Clin Invest 1981;68:1305-1313.

13. Ravdin JI, Murphy CF, Salata RA, Guerrant RL, Hewlett EL. N-acetyl-D-galactosamine-inhibitable adherence lectin of *Entamoeba histolytica*: partial purification and relation to amebic virulence *in vitro*. J Infect Dis 1985;151:804-815.

14. Ravdin JI, Moreau F, Sullivan JA, Petri Jr. WA, Mandell GL. Relationship of free intracellular calcium to the cytolytic activity of *Entamoeba histolytica.* Infect Immun 1988;56:1505-1512.

15. Que X, Reed SL. The role of extracellular cysteine proteinases in the pathogenesis of *Entamoeba histolytica* invasion. Parasitology Today 1997;13:190-194.

16. Long-Krug SA, Fischer KJ, Hysmith RM, Ravdin JI. Phospholipase A enzymes of *Entamoeba histolytica*: description and subcellular localization. J Inf Dis 1985;152:536-541.

17. Leippe M, Andra J, Muller-Eberhard HJ. Cytolytic and antibacterial activity of synthetic peptides derived from amoebapore, the pore-forming peptide of *Entamoeba histolytica.* Proc Natl Acad Sci USA 1994;91:2602-2606.

18. McGowan K, Piver G, Stoff JS, Donowitz M. Role of prostaglandins and calcium in the effects of *Entamoeba histolytica* on colonic electrolyte transport. Gastroenterology 1990;98:873-880.

19. Shibayama M, Navarro-Garcia F, Lopez-Revilla R, Martinez-Palomo A, Tsutsumi V. *In vivo* and *in vitro* experimental intestinal amebiasis in Mongolian gerbils (*Meriones unguiculatus*) Parasitol Res 1997;83:170-176.

20. Prathap K, Gilman R. The histopathology of acute intestinal amebiasis. A rectal biopsy study. Am J Pathol 1970;60:229-246.

21. Chadee K, Meerovitch E. *Entamoeba histolytica*: early progressive pathology in the cecum of the gerbil (*Meriones unguiculatus*). Am J Trop Med Hyg 1985;34:283-291.

22. Yu Y, Chadee K. *Entamoeba histolytica* stimulates interleukin-8 from human colonic epithelial cell without parasite-enterocyte contact. Gastroenterology 1997;112:1536-1547.

23. Baggiolini M, Dewbald B, Moser B. Interleukin-8 and related chemotactic cytokines--CXC and CC chemokines. Adv Immunol 1994;55:97-197.

24. MacDermott RP. Chemokines in the inflammatory bowel diseases. J Clin Immunol 1999;19:266-72.

25. Chadee K, Keller K, Forstner J, Innes DJ, Ravdin JI. Mucin and nonmucin secretagogue activity of *Entamoeba histolytica* and cholera toxin in rat colon. Gastroenterology 1991;100:986-997.

26. Chadee K, Meerovitch E. The Mongolian gerbil (*Meriones unguiculatus*) as an experimental host for *Entamoeba histolytica*. Am J Trop Med Hyg 1984;33:47-54.

27. Stanley Jr. SL, Jackson TFHG, Reed SL, Calderon J, Kunz-Jenkins C, Gathiram V, Li E. Serodiagnosis of invasive amebiasis using a recombinant *Entamoeba histolytica* protein. JAMA 1991;266:1984-1986.

28. Soong C-J, Kain KC, Abd-Alla M, Jackson TFHG, Ravdin JI. A recombinant cystein-rich section of the *Entamoeba histolytica* galactose-inhibitable lectin is efficacious as a subunit vaccine in the gerbil model of amebic liver abscess. J Infect Dis 1995;171:645-651.

29. Petri Jr. WA, Broman J, Healy G, Quinn T, Ravdin JI. Antigenic stability and immunodominance of the Gal/GalNAc adherence lectin of *Entamoeba histolytica*. Am J Med Sci 1989;296:163-165.

30. Espinosa-Cantellano M, Martinez-Palomo A. *Entamoeba histolytica*: mechanism of surface receptor capping. Exp Parasitol 1994;79:424-435.

31. Reed SL, Curd JG, Gigli I, Gillin FD, Braude AI. Activation of complement by pathogenic and nonpathogenic *Entamoeba histolytica*. J Immunol 1986;136:2265-2270.

32. Braga LL, Ninomiya H, McCoy JJ, Eacker S, Wiedmer T, Pham C, Wood S, Sims PJ, Petri Jr. WA. Inhibition of the complement membrane attack complex by the galactose-specific adhesin of *Entamoeba histolytica*. J Clin Invest 1992;90:1131-1137.

33. Palmer RB. Changes in the liver in amebic dysentery, with special reference to the

origin of amebic abscess. Arch Pathol 1938;25:327-335.

34. Brandt H, Tamayo RP. Pathology of human amebiasis. Human Pathol 1970;1:351-385.

35. Tsutsumi V, Mena-lopez R, Anaya-Velazquez F, Martinez-Palomo A. Cellular bases of experimental amebic liver abscess formation. Am J Pathol 1984;117:81-91.

36. Chadee K, Meerovitch E. The pathogenesis of experimentally induced amebic liver abscess in the gerbil (*Meriones unguiculatus*). Am J Pathol 1984;117:71-80.

37. Ghadirian E, Meerovitch E. Effect of immunosuppression on the size and metastasis of amebic liver abscesses in hamsters. Parasite Immunol 1981;3:329-338.

38. Salata RA, Martinez-Palomo A, Murray HW, Canales L, Trevino N, Sgovia E, Murphy CF, Ravdin JI. Patients treated for amebic liver abscess develop cell-mediated immune responses effective *in vitro* against *Entamoeba histolytica*. J Immunol 1986;136:2633-2639.

39. Salata RA, Cox JG, Ravdin JI. The interaction of human T-lymphocytes and *Entamoeba histlolytica*: killing of virulent amebae by lectin-dependent lymphocytes. Parasite Immunol 1987;9:249-261.

40. Salata RA, Murray HW, Rubin BY, Ravdin JI. The role of gamma interferon in the generation of human macrophages cytotoxic for *Entamoeba histolytica* trophozoites. Am J Trop Med Hyg 1987;37:72-78.

41. Denis M, Chadee K. Cytokine activation of murine macrophages for *in vitro* killing of *Entamoeba histolytica* trophozoites. Infect Immun 1998;57:1750-1756.

42. Ghadirian E, Salimi A. In vitro effect of recombinant interferon gamma in combination with LPS on amebicidal activity of murine Kupffer cells. Immunobiol 1993;188:203-219.

43. Denis M, Chadee K. Human neutrophils activated by interferon-γ and tumour necrosis factor-α kill *Entamoeba histolytica* trophozoites *in vitro*. J Leukoc Biol 1989;46:270-274.

44. Lin J-Y, Chadee K. Macrophage cytotoxicity against *Entamoeba histolytica* trophozoites is mediated by nitric oxide from L-arginine. J Immunol 1992;148:3999-4005.

45. Campbell D, Chadee K. Interleukin (IL)-2, IL-4, and tumor necrosis factor-α responses during *Entamoeba histolytica* liver abscess development in gerbils. J Inf Dis 1997;175:1176-1183.

46. Denis M, Chadee K. *In vitro* and *in vivo* studies of macrophage functions in amebiasis. Infect Immun 1988;56:3126-3131.

47. Wang W, Keller K, Chadee K. Modulation of tumor necrosis factor production by macrophages in *Entamoeba histolytica* infection. Infect Immun 1992;60:3169-3174.

48. Wang W, Chadee K. *Entamoeba histolytica* suppresses gamma interferon-induced macrophage class II major histocompatibility complex Ia molecule and I-Aß mRNA expression by a prostaglandin E_2-dependent mechanism. Infect Immun 1995;63:1089-

1094.

49. Gill NJ, Ganguly NK, Dilawari JB, Mahajan RC. Lymphocyte subpopulations transformation studies in an experimental model of intestinal and hepatic amoebiasis. Trans R Soc Trop Med Hyg 1985;79:618-623.

50. Gandhi BM, Gupta H, Irshad M, Chawala TC, Tandon BN. Cell-mediated immunity in amoebic liver abscess. J Trop Med Hyg 1986;89:163-166.

51. Salata RA, Martinez-Palomo A, Canales L, Murray HW, Trevino N, Ravdin JI. Supression of T-lymphocyte responses to *Entamoeba histolytica* antigen by immune sera. Infect Immun 1990;58:3941-3946.

52. Wang W, Chadee K. *Entamoeba histolytica* alters arachidonic acid metabolism in macrophages *in vitro* and *in vivo*. Immunology 1992;76:242-250.

53. Sanchez-Ramirez B, Escalante B, Rosales-Encina JL, Talamas-Rohana P. Role of prostaglandin E_2 in amebic liver abscess formation in hamsters. Prostaglandins 1997;53:411-421.

Chapter 2. Intestinal mucins[1]

Intestinal mucins are high molecular weight glycoproteins that provide the structural component to mucus and protect the mucosal epithelium from pathogens, chemical irritants and mechanical stress. The biochemistry of intestinal mucins confers their protective nature: the protein backbone has a high O-linked oligosaccharide content (>80% carbohydrate by mass; Figure 1A) that provides lectin binding capacity whereas the ability of the protein core to form multimers (through disulphide bonds) and polymerize into gels bestows viscoelasticity to the mucous blanket (Figure 1B).

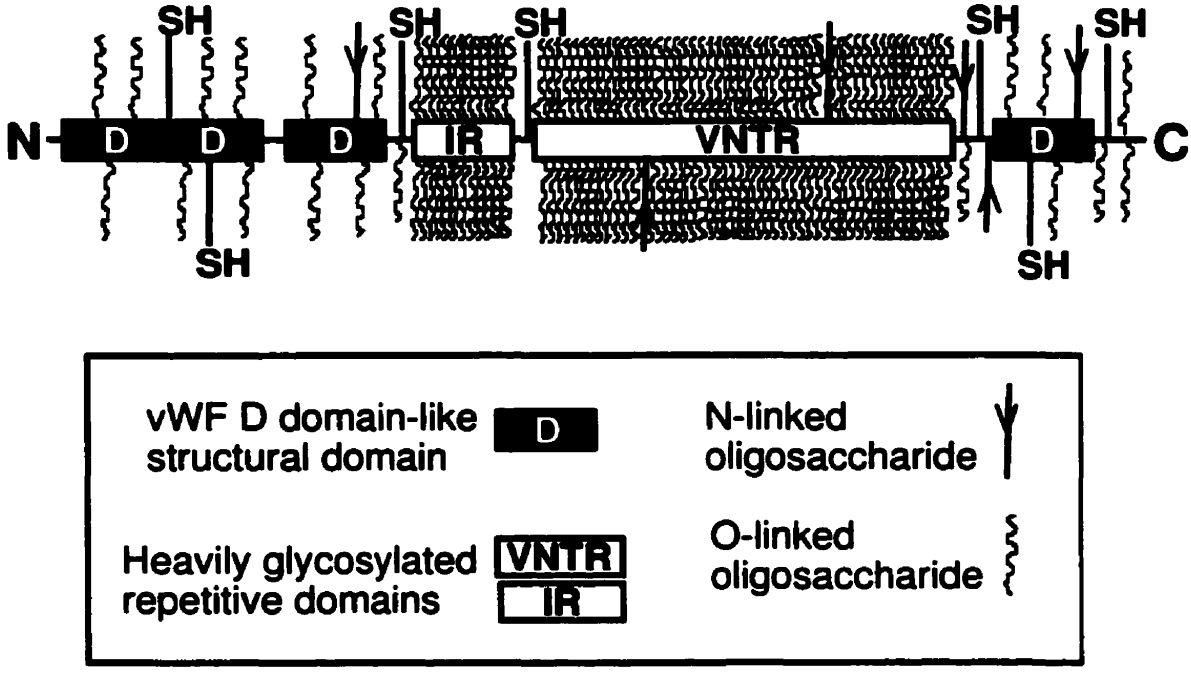

Figure 1A. Schematic representation of MUC2. (adapted from reference 1)

[1]Portions of this Chapter were adapted from the following reference:

Belley A, Keller K, Goettke M, Chadee K. Intestinal mucins in colonization and host defense against pathogens. Am J Trop Med Hyg 1999;60:10-15 (supplement).

Figure 1B. Hypothetical model of a MUC2 polymer. The sulfhydryl bonds at the carboxy- and amino-terminal ends link two MUC2 monomers to form a polymeric structure. The heavily O-glycosylated regions (VNTR and IR) are depicted by the bottle brush areas (adapted from reference 1).

2.1 The goblet cell

Goblet cells are exocrine cells of the intestinal epithelium specialized in mucin exocytosis. Mucins are secreted constitutively in what has been termed "unregulated" secretion or in response to secretagogues in a "regulated" fashion (2). The uniqueness of these cells is their ability to store membrane bound mucin granules within an intracellular storage compartment known as the theca. The theca lies below the apical membrane and is made up of microtubules and intermediate filament bundles intermeshed together in a basket-like structure (3). Overlying the theca is a network of F-actin filaments which is believed to be a physical barrier that prevents contact between granules and the plasma membrane. Accordingly, disruption of F-actin filaments with cytochalasin D accelerated the movement of granules through the apical granule mass and caused the release of small amounts of stored mucins (4, 5). Baseline secretion of mucins involves the release of newly synthesized mucins at the apical membrane. Granules destined for immediate release are guided to the membrane by microtubules. This process can be inhibited by the microtubule depolymerizing agent nocodazole and by monensin, an inhibitor of Golgi function (4). It is unknown how mucins are targeted for immediate secretion or storage.

2.2 Exocytosis

Little is known about the exocytotic ``machinery'' involved in regulated mucin exocytosis. The majority of the literature dealing with exocytosis focuses on the release of neurotransmitters by neuronal cells and insulin secretion by pancreatic ß-cells. These studies distinguish between synaptic vesicles that carry neurotransmitters and large dense core vesicles (LDCV) that carry for example, the peptide hormone insulin (6). It would seem likely that the release of preformed mucins reflects more the process of insulin exocytosis than neurotransmitters based on the nature of the granule content. However, several machinery proteins appear to be common to both cell types. The SNARE proteins, initially regarded as neuron-specific, have been identified in insulin-secreting ß-cells (7, 8). The SNARE hypothesis is a generalized theory for regulated exocytosis and includes four key components (9): 1) a vesicle membrane protein termed v-SNARE (synaptobrevin), 2) a target membrane protein termed t-SNARE (a complex of syntaxin 1a/b and SNAP-25), 3) a cytosolic ATPase termed N-ethylmaleimide-sensitive fusion protein (NSF) required for membrane fusion, and 4) an adaptor protein termed SNAP (α, ß or γ) for NSF that enables binding to the v-SNARE/t-SNARE complex. Once docking has occurred (i.e., synaptobrevin/syntaxin-SNAP-25 binding), binding of NSF-SNAP to the complex is ultimately required for membrane fusion. Recently, a Ca^{2+} sensory vesicle protein termed synaptotagmin I was shown to interact directly with ß-SNAP (the brain SNAP isoform) and SNAP-25 and may be the Ca^{2+}-triggering mechanism required for vesicle fusion to the plasma membrane (10). Furthermore, interaction between the t-SNARE syntaxin and a juxtaposed membrane Ca^{2+} channel has been shown (11-13). It was proposed that a Ca^{2+} channel would need to be in close proximity to supply the needed concentration of Ca^{2+} ions to the reaction, which appears to be the case.

Independent of the SNARE mechanism of exocytosis is the ability of guanine

nucleotides and guanine nucleotide binding proteins (GTP-binding proteins or G proteins) to stimulate exocytosis. Application of GTP or its slowly hydrolyzed counterpart GTPγS to ß-cells, chromaffin cells and neutrophils induced exocytosis (14, 15, 16). Activation of granule-associated $G_{\alpha o}$ regulated actin microfilament depolymerization and inhibited catecholamine secretion by chromaffin cells in response to Ca^{2+} (17). Moreover, the Ras-related small GTP-binding protein Rab4 was shown to negatively modulate exocytosis of amylase by rat pancreatic acini (18). In contrast, $G_{\beta\gamma}$ subunits applied to permeabilized mast cells prevents the run-down (leaking of soluble factors involved in exocytosis that leads to a refractory period) in Ca^{2+} and GTPγS stimulated cells and prolongs hexosaminidase secretion (19).

2.3 Mucin biosynthesis

Of the nine human mucin genes identified, the prominent secretory mucin within the intestinal tract is encoded by the *MUC2* gene (20) (Table 1). MUC2 is produced by goblet cells of the small and large intestine (22). The *MUC2* cDNA spans 15,563 bp and codes for a protein larger than 5000 amino acids with distinct peptide domains (23). The amino- and carboxy-terminal domains are cysteine rich and are involved in the polymerization of MUC2 (see Figure 1A). Also within each termini are D domains, which share high identity with those of pro-von Willibrand factor, a polymeric serum glycoprotein (Figure 1). The D domains are thought to be involved in polymerization (1). The central region of the molecule is highly O-glycosylated and contains a variable number (40 to 115) of tandem repetitive units (VNTR) consisting mainly of a 23 amino acid sequence of threonine and proline residues (PTTTPITTTTTVTPTPTPTGTQT). Flanking the central region is an irregular repeat (IR) domain containing variations of a 16 amino acid sequence

(PSPPPTTTTTPPPTTT) that is also heavily O-glycosylated (2).

Table 1. Tissue distribution of human secreted mucins

Mucin	Tissue/cell expression
MUC2	Colon and small intestine (goblet cells), salivary gland ducts, inferior turbinates
MUC5AC	Colon (goblet cells), superficial stomach epithelium, bronchus (mucous glands and ciliated epithelium), endocervical epithelium, inferior turbinates
MUC5B	Salivary glands, submandibular glands, gall bladder biliary epithelial cells, bronchus (mucous and serous glands), colon (goblet cells), endocervical epithelium, inferior turbinates (submucosal glands)
MUC6	Colon, small intestine (goblet cells), gall bladder epithelium, epithelium of stomach (mucous neck cells; antral mucous cells), seminal vesicle, pancreas (centroacinar cells and ducts), endocervical epithelium, endometrial epithelium, biliary epithelial cells
MUC7	Salivary glands (mucous cells), bronchial airways (submucosal glands), inferior turbinates (submucosal glands)

Adapted from reference 21

Another intestinal mucin, MUC3, is expressed by goblet and absorptive cells (22). It also contains tandem repetitive units rich in serine and threonine that are heavily O-glycosylated (24). The functional relevance of MUC3 is unclear: its cellular distribution suggests that it may not be secreted and may not contribute significantly to the colonic mucous blanket (25).

A typical feature of mucin mRNA is a polydisperse signal on Northern blots. Only recently was the polydispersity shown to result from shearing of the mucin mRNAs during extraction (26). An improved isolation technique that reduced shear forces during extraction revealed that the *MUC2* and *MUC3* mRNAs are extremely large and estimated to be approximately 16 kb and 17 kb, respectively. Moreover, the transcripts for both genes

were determined to be stable with half-lives of approximately 13 hours.

A number of groups have studied the biosynthesis of MUC2 (27-29). MUC2 apoprotein is core N- (cotranslationally) and O-glycosylated (only GalNAc residues) early within the endoplasmic reticulum (28). Oligomerization (or perhaps dimerization) of subunits follows, a process which is ultimately required so that entry into the Golgi can occur (2). Monomers are presumably dimerized in a "tail to tail" (carboxy-termini) fashion through disulphide bonds (1). Recent studies demonstrated that most of the MUC2 mucins from cultured cells and human colon were present in oligomeric form in an insoluble pellet after guanidinium chloride extraction (30, 31). Following reduction with dithiothreitol, MUC2 monomers were detected along with dimers that were insensitive to reduction. The nature of the novel nonreducible intermolecular bond was not determined. Another study showed that monensin, an agent that disrupts Golgi activity, did not affect oligomerization of mucin precursors but instead decreased the incorporation of ^{3}H-glucosamine into oligomeric mucins of the LS180 cell line (27). The bulk of the O-linked oligosaccharides are believed to be added to core sugars (chain elongation) within the Golgi apparatus. Oligomeric mucins bud from the trans-Golgi face within membrane bound condensing granules and accumulate in the theca. Oligomeric mucins are further multimerized in a "head to head" (amino-termini) fashion, possibly within mucin granules, yielding fully mature molecules (1, 29) (see Figure 1B).

2.4 *MUC2* gene structure

The 5'-promoter sequence of the *MUC2* gene has been sequenced and analysis of the region revealed a TATA box and several potential response element sequences (Figure 2): $[cAMP]_i$ responsive element (CRE), NF-κB, tumor responsive element (TRE), AP2,

five Adh1 sites upstream of the NF-κB site, CACCC box, and C/EBP (32, 33).

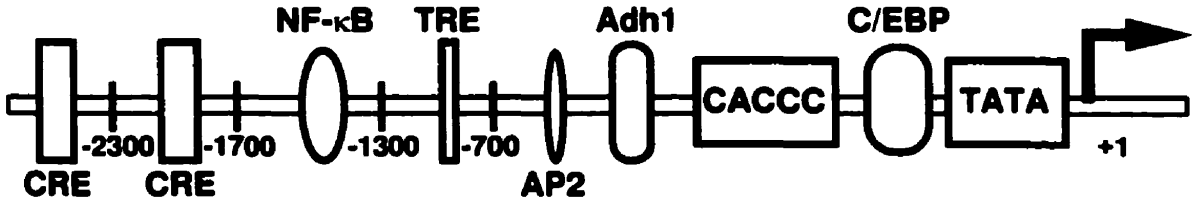

Figure 2. Diagram of the 5'-promoter region of the human *MUC2* gene. The arrow indicates the transcription start site and numbers show the base pairs relative to the initiation site. Note only one Adh1 site is shown for demonstrative purposes only.

The CACCC box located between -91 to -73 base pairs (bp) was shown to be a major controlling factor for constitutive expression of *MUC2* in transient transfections of luciferase reporter gene constructs. It was shown that Sp1 and other Sp1 family members bind to this motif. Moreover, the region between -228 to -178 increased expression in *MUC2* expressing cell lines but not in non-expressing cell lines (32). Analysis of this sequence revealed no known nuclear protein-binding sites but otherwise indicates that an element in this region is important for specific expression of *MUC2*. To further demonstrate the presence of tissue-specific regulators within the *MUC2* promoter, a construct spanning -2864 to +17 bp of the *MUC2* promoter was fused 5' to the human growth hormone reporter gene (34). Transgenic mice expressing the recombinant reporter construct were generated. Reporter gene expression was detected in the distal small intestine in four of eight transgenic lines. Expression of the reporter construct may be highly dependent on the site of insertion into the genome and may explain why four lines incorporated the construct but did not express it. Only one line expressed the reporter construct in the colon. Although the expression levels of the reporter construct differed from normal *MUC2*, elements required for expression in the small intestine lie within -

2864 to +17 bp of the promoter. It was suggested that elements lying outside this region may control colonic tissue expression (34).

2.5 Transcriptional and posttranscriptional regulation of *MUC2*

In contrast to secretagogue-induced release of preformed (stored) mucins, the constitutive release of mucins involves the translocation of newly synthesized mucins budding from the trans-Golgi face to the apical membrane. This process is dependent on the rate of synthesis and therefore gene expression (i.e. an increase in the transcriptional rate increases constitutive secretion) (35): it seems plausible that increased *MUC2* gene expression may translate to increased secretion of MUC2 but in contrast, it may be a mechanism whereby the cell replenishes its stored mucin pooled quickly following exocytosis. Clearly more work is needed to understand the significance of increased gene expression and its effect on mucin exocytosis.

A few studies have addressed how *MUC2* is regulated transcriptionally and posttranscriptionally. Upregulation of the *MUC2* gene occurs in the airways of cystic fibrosis patients. A recent study showed that lipopolysaccharide (LPs) from *Pseudomonas aeruginosa* increases the expression of *MUC2* in transient transfection experiments of the *MUC2* promoter (36). A follow-up study showed that *P. aeruginosa* activates the c-Src-Ras-MEK1/2-MAPK-pp90srk signaling pathway to promote NF-κB binding to the NF-κB site at -1452 to -1441 bp of the *MUC2* promoter (37). Exoproducts from the bacterium also increased expression of the airway mucin gene *MUC5AC* (38). Tumor necrosis factor-α (TNF-α) stimulated MUC2 hypersecretion in human airway organ cultures and the human pulmonary mucoepidermoid carcinoma cell line NCI-H292 (39). Moreover, it caused the accumulation of *MUC2* mRNA. The $[cAMP]_i$ elevating agent forskolin and the protein kinase C (PKC) activating agent 12-O-tetradecanoylphorbol-13-acetate caused the

accumulation of *MUC2* mRNA in human colonic HT29 cells differentiated *in vitro* to the goblet cell lineage (40). These agents did not initiate transcription as measured by nuclear run-off assays and therefore regulate *MUC2* expression posttranscriptionally. However, in another study it was shown that activation of PKC induced *MUC2* mRNA gene transcription (35). Cholera toxin, a $[cAMP]_i$ elevating agent, not only increased mucin secretion but also caused the accumulation of *MUC2* mRNAs in the HT-29 Cl.16E cell line (41). Moreover, retinoic acid increased *MUC2* mRNA accumulation in normal human bronchial epithelial (NHTBE) cells (42).

2.6 Mucin secretagogues and signal transduction

A large body of work has focused on identifying mucin secretagogues to gain insight on what causes regulated mucin exocytosis. Neurotransmitters, inflammatory mediators and chemical agents induce mucin release from the stored mucin pool (Table 2). Mucin secretagogues have been shown to signal through several second messengers including intracellular cyclic adenosine monophosphate ($[cAMP]_i$), intracellular Ca^{2+} ($[Ca^{2+}]_i$) or diacylglycerol (for the activation of protein kinase C). Stimulated release may involve either the gradual release of stored mucins or compound exocytosis, where centrally located granules fuse consecutively with the apical plasma membrane and discharge their contents. Goblet cells having undergone compound exocytosis appear cavitated within the apical granular region under microscopy.

Activation of protein kinases by second messengers appears to be an important step in mucin exocytosis. The protein kinase A (PKA) inhibitor peptide and the PKC inhibitors H7 and bisindolylmaleimide 1 inhibited forskolin- and PMA-induced secretion, respectively (41, 55, 56). Furthermore, the broad spectrum protein kinase inhibitor

staurosporine inhibited both PMA- and forskolin-induced secretion. Evidence indicates that the PKC-ε isoform mediates PMA driven mucin exocytosis in HT-29/A1, T84 and LS180 colonic cells (55). Interestingly, stimulation of mucin secretion simultaneously with PMA and the adenylate cyclase activator forskolin, enhanced secretion beyond that of forskolin or PMA alone but was less than additive (56). This may suggest that secretion was stimulated near maximally by either agent or because the response was less than additive, the kinases may phosphorylate the same cellular effectors (exocytosis machinery proteins).

Table 2. Summary of gastrointestinal mucin secretagogues

Secretagogue	Second messenger/kinase	Receptor-mediated	Tissue/cell line	References
Dibutyryl cAMP	$[cAMP]_i$*	No	HT29	41
Forskolin	$[cAMP]_i$	No	HT29, T84, LS174T	43, 44
Cholera toxin	$[cAMP]_i$	No	HT29, rat small intestine and colon	41, 43, 45, 46
Vasoactive intestinal peptide	$[cAMP]_i$†	Yes	HT29, T84	44, 47
PGE_2	$[cAMP]_i$	Yes	Rabbit and rat gastric cells, rat colon, HT29, T84, LS174T	44, 48, 49
Serotonin	$[cAMP]_i$†	Yes‡	Rat small intestine	50
Carbachol	$[Ca^{2+}]_i$	Yes	HT29, T84	44, 51
Neurotensin	$[Ca^{2+}]_i$	Yes	HT29	51
Ionophores	$[Ca^{2+}]_i$	No	Rabbit stomach, HT29, T84, LS174T, LS180, SW1116	4, 44, 52-54
Phorbol ester	PKC§	No	HT29, T84, LS174T, LS180	4, 44, 52

*Membrane permeable $[cAMP]_i$ mimetic. †Based on the second messenger that the receptor activates. § Phorbol esters mimic the second messenger diacylglycerol and directly activate PKC.

Following kinase activation, it is unknown how phosphorylation regulates mucin exocytosis. Some interesting targets of phosphorylation that may directly regulate exocytosis are the SNARE proteins. Phosphorylation of SNAP-25 by PKC in permeabilized PC12 cells enhanced the secretion of norepinephrine (57). Other SNARE proteins have been shown to be phosphorylated but how this affects exocytosis is still under investigation (58).

2.7 Mucins in disease

Altered glycosylation and aberrant mucin gene expression occur during various gastrointestinal disease states. Human colon cancer cells with high metastatic potential secrete large quantities of MUC2 mucins. Increased metastatic potential of primary tumors in nude mice occurs due to the enhanced expression of sialylated core carbohydrates by subpopulations of colon cancer cells (the T or Tn antigens are replaced by sialyl T or sialyl Tn) (59). Recently, it was shown that metastatic potential was decreased in the metastatic colon cancer cell line LS LiM6 stably expressing a *MUC2* antisense construct (60). Expression of the antisense mRNA decreased MUC2 protein production by 50% and caused a marked reduction in liver colonization.

Aside from modifications of carbohydrate structures, cancerous tissues express mucin genes not ordinarily expressed in normal tissues. For example, normal gastric tissues have undetectable levels of MUC2 and MUC3 whereas in gastric intestinal metaplasia, there is aberrant secretion of both these mucins (61). Furthermore, colonic adenocarcinomas produced higher amounts of MUC2 and MUC3 when compared to normal tissues. The greatest increase of *MUC2* gene expression occurs in mucinous colon cancer. It is unknown why aberrant gene expression occurs or whether it plays a role in pathology.

Although it is easy to suggest that altered mucin secretion or glycosylation may exacerbate symptoms of inflammatory bowel disease (IBD), it is unknown whether mucins play a role in the pathogenesis of IBD. Several alterations occur in IBD that are not seen in normal colon. Mucous gel thickness was decreased in IBD and was shown to harbor high numbers of bacteria (62, 63). MUC2 biosynthesis and secretion was decreased in biopsy specimens from patients with ulcerative colitis (64, 65). Upon remission, the biosynthetic rate and secretion of MUC2 returned to control levels. Shortening of mucin O-linked carbohydrate chains was demonstrated in ulcerative colitis which may weaken their protective function (66).

2.8 Interaction between mucins and intestinal microorganisms

A number of microorganisms have been found to bind to mucin carbohydrate moieties. A summary of various mucin-microbial interactions is shown in Table 3.

Table 3. Specific interaction between pathogen adhesins and intestinal mucin components

Pathogen	Microbial receptor	Mucin ligand	References
Rotavirus	Outer capsid proteins (?)	Sialic acid	67
Campylobacter jejuni	Flagella lipopolysaccharide	Fucose	68
Escherichia coli	Type 1 pili K88ab fimbriae K99, F41, Fy pili	N-linked glycan (mannose) GalNAc Saccharides	69-72
Salmonella typhimurium	66 kDa heat shock protein	?	73
Shigella sonnei *Shigella boydii*	?	Carbohydrate	74
Vibrio cholerae	?	Fucose	75
Yersinia enterocolitica	?	Gal/GalNAc	76, 77
Entamoeba histolytica	Gal lectin	Gal/GalNAc	78

Protection of the intestinal epithelium against pathogenic microorganisms lies in the binding capacity of mucin carbohydrates to microbial adhesins. Binding of microorganisms to mucins prevents their attachment to the epithelium and serves as the first line of host defense against invasion. The fate of mucin-bound microorganisms is dependent on their inherent ability to colonize the intestinal tract (Table 4). Many organisms are sloughed and swept out during peristaltic movements and defecation. Nonpathogenic organisms such as the indigenous intestinal flora that reside in the adherent mucous blanket occupy an important niche within the intestines; they prevent the attachment of pathogenic organisms by occupying available binding sites. Once bound, pathogenic microorganisms must either colonize and/or penetrate to the mucosal layer. Pathogens may secrete virulence factors such as cytotoxins, cytolysins or invasins. They may also release secretagogues. It is unknown what effect prolonged exposure to microbial secretagogues might have on intestinal goblet cells: the persistence of the secretagogue may eventually deplete mature mucin stores by causing the secretion of immature mucins (poorly glycosylated or subunit mucins). The latter condition could result in mucins with a decreased lectin binding or gel

Table 4. Possible outcomes in the interaction between pathogens and intestinal mucins

Microbial action	Potential outcome of mucin-bound microbes
Initial mucin binding	Eliminated through sloughing and peristalsis
Colonization	Retained in mucous blanket (colonization only): access denied to underlying epithelium
Colonization	"Tracking" toward epithelium: secretion of virulence factors
Epithelial invasion	Mucous barrier breached: invasion of the intestinal epithelium

forming capacity, respectively. Another possible microbial mechanism to overcome the mucous layer is to secrete proteinases, which may target and cleave the poorly glycosylated

regions of the MUC2 molecule. These regions have been shown to be susceptible to cleavage with trypsin and once cleaved, may destroy the polymeric structure of luminal mucins and render the mucus less protective (31).

2.9 References

1. Gum Jr. JR. Human mucin glycoproteins: varied structures predict diverse properties and specific functions. Biochem Soc Trans 1995;23:795-799.

2. Forstner JF, Forstner GG. Gastrointestinal mucus. In Johnson LR, ed. Physiology of the gastrointestinal tract. Volume 2, 3rd ed. New York: Raven, 1995:1255-1283.

3. Specian RD, Oliver MG. Functional biology of intestinal goblet cells. Am J Physiol 1991;260:C183-C193.

4. McCool DJ, Forstner JF, Forstner GG. Regulated and unregulated pathways for MUC2 mucin secretion in human colonic LS180 adenocarcinoma cells are distinct. Biochem J 1995;312:125-133.

5. Oliver MG, Specian RD. Cytoskeleton of intestinal goblet cells: role of actin filaments in baseline secretion. Am J Physiol 1990;259:G9910-G997.

6. Lang, J. Molecular mechanisms and regulation of insulin exocytosis as a pardigm of endocrine function. Eur. J. Biochem. 1999;259:3-17.

7. Jacobsson G, Bean AJ, Scheller RH, Juntti Berggren L, Deeney JT, Berggren PO,

Meister B. Identification of synaptic proteins and their isoform mRNAs in compartments of pancreatic endocrine cells. Proc Natl Acad Sci USA 1994;91:12487-12491.

8. Kiraly-Borri C, Morgan A, Burgoyne R, Weller U, Wollheim C, Lang J. Soluble N-ehtylmaleimide sensitive factor attachment protein and N-ehtylmaleimide insensitive factors are required for Ca^{2+} stimulated exoctosis of insulin. Biochem J 1996;314:199-203.

9. Ferro-Novick S, Jahn R. Vesicle fusion from yeast to man. Nature 1994;370:191-193.

10. Rizo J, Südhof TC. C2-domains, structure and function of a universal Ca^{2+}-binding domain. J Biol Chem 1998; 273:15879-15883.

11. Bezprozvanny I, Scheller RH, Tsien RW. Functional impact of syntaxin on gating of N-type and Q-type calcium channels. Nature 1995;378:623-626.

12. Sheng ZH, Rettig J, Cook T. Catterall WA. Calcium-dependent interaction of N-type calcium channels with the synaptic core complex. Nature 1996; 379:451-454.

13. Lang J, Fukuda M, Zhang H, Mikoshiba K, Wollheim CB. The first C2 domain of synaptotagmin is required for exocytosis of insulin from pancreatic ß-cells: action of synaptotagmin at low micromolar calcium. EMBO J 1997;16:4591-4596.

14. Vallar L, Biden TJ, Wollheim CB. Guanine nucleotides induce Ca^{2+}-independent insulin secretion from permeabilized RINm5F cells. J Biol Chem 1987;262:5049-5056.

15. Barrowman MM, Cockcroft S, Gomperts BD. Two roles for guanine nucleotides in the stimulus-secretion sequence of neutrophils. Nature 1986;319:504-507.

16. Bittner MA, Holz RW, Neubig RR. Guanine nucleotide effects on catecholamine secretion from digitonin-permeabilized adrenal chromaffin cells. J Biol Chem 1986;261;10182-10188.

17. Gasman S, Chasserot-Golaz S, Popoff MR, Aunis D, Bader MF. Trimeric G proteins control exocytosis in chromaffin cells. G_o regulates the peripheral actin network and catecholamine secretion by a mechanism involving the small GTP-binding protein Rho. J Biol Chem 1997;272:20564-20571.

18. Ohnishi H, Mine T, ShibataH, Ueda N, TsuchidaT, Fujita T. Involvement of Rab4 in regulated exocytosis of rat pancreatic acini. Gastroenterology 1999;116:943-952.

19. Pinxteren JA, O'Sullivan AJ, Tatham PER, Gomperts BD. Regulation of exocytosis from rat peritoneal mast cells by G protein subunits. EMBO J 1998;17:6210-6218.

20. Tytgat KMAJ, Buller HA, Opdam FJM, Kim YS, Einerhand AWC, Dekker J. Biosynthesis of human colonic mucin: MUC2 is the prominent secretory mucin. Gastroenterology 1994;107:1352-1363.

21. Perez-Vilar J, Hill RL. The structure and assembly of secreted mucins. J Biol Chem 1999;274:31751-31754.

22. Chang SK, Dohrman AF, Basbaum CB, Ho SB, Tsuda T, Toribara NW, Gum JR, Kim YS. Localization of mucin (MUC2 and MUC3) messenger RNA and peptide expression in human normal intestine and colon cancer. Gastroenterology 1994;107:28-36.

23. Gum Jr. JR, Hicks JW, Toribara NW, Siddiki B, Kim YS. Molecular cloning of the human intestinal mucin (MUC2) cDNA. J Biol Chem 1994;269:2440-2446.

24. Gum Jr. JR, Hicks JW, Swallow DM, Lagace RE, Byrd JC, Lamport DTA, Siddiki B, Kim YS. Molecular cloning of cDNAs derived from a novel human intestinal mucin gene. Biochem Biophys Res Commun 1990;171:407-415.

25. Gum Jr. JR, Ho JJL, Pratt WS, Hicks JW, Hill AS, Vinall LE, Roberton AM, Swallow DM, Kim YS. MUC3 human intestinal mucin. J Biol Chem 1997;272:26678-26686.

26. Debailleul V, Laine A, Huet G, Mathon P, d'Hooghe MC, Aubert JP, Porchet N. Human mucin genes MUC2, MUC3, MUC4, MUC5AC, MUC5B, and MUC6 express stable and extremely large mRNAs and exhibit a variable length polymorphism. J Biol Chem 1998;273:881-890.

27. McCool DJ, Forstner JF, Forstner GG. Synthesis and secretion of mucin by the colonic tumor cell line LS180. Biochem J 1994;302:111-118.

28. Sheehan JK, Thornton DJ, Howard M, Carlstedt I, Corfield AP, Paraskeva C. Biosynthesis of the MUC2 mucin: evidence for a slow assembly of fully glycosylated

units. Biochem J 1996;315:1055-1060.

29. McGuckin MA, Devine PL, Ward BG. Early steps in the biosynthesis of MUC2 epithelial mucin in colon cancer cells. Biochem Cell Biol 1996;74:87-93.

30. Axelsson MAB, Asker N, Hansson GC. O-glycosylated MUC2 monomer and dimer from LS174T cells are water-soluble, whereas larger MUC2 species formed early during biosynthesis are insoluble and contain nonreducible intermolecular bonds. J Biol Chem 1998;273:18864-18870.

31. Herrmann A, Davies JR, Lindell G, Martensson S, Packer NH, Swallow DM, Carlstedt I. Studies on the "insoluble" glycoprotein complex from human colon. Identification of reduction-insensitive MUC2 oligomers and C-terminal cleavage. J Biol Chem 1999;274:15828-15836.

32. Gum Jr. JR, Hicks JW, Kim YS. Identification and characterization of the MUC2 (human intestinal mucin) gene 5'-flanking region: promoter activity in cultured cells. Biochem J 1997;325:259-267.

33. Velcich A, Palumbo L, Selleri L, Evans G. Organization and regulatory aspects of the human intestinal mucin gene (MUC2) locus. J Biol Chem 1997;272:7968-7976.

34. Gum Jr. JR, Hicks JW, Gillespie A-M, Carlson EJ, Komuves L, Karnik S, Hong JC, Epstein CJ, Kim YS. Goblet cell-specific expression mediated by the *MUC2* mucin gene promoter in the intestine of transgenic mice. Am J Physiol 1999;276:G666-G676.

35. Hong D-H, Petrovics G, Anderson WB, Forstner J, Forstner G. Induction of mucin gene expression in human colonic cell lines by PMA is dependent on PKC-ε. Am J Physiol 1999;277:G1041-G1047.

36. Li JD, Dohrman AF, Gallup M, Miyata S, Gum JR, Kim YS, Nadel JA, Prince A, Basbaum CB. Transcriptional activation of mucin by *Pseudomonas aeruginosa* lippopolysaccharide in the pathogenesis of cystic fibrosis lung disease. Proc Natl Acad Sci USA 1997;94:967-972.

37. Li JD, Feng W, Gallup M, Kim J-H, Gum J, Kim Y, Basbaum C. Activation of NF-κB via a Src-dependent Ras-MAPK-pp90rsk pathway is required for *Pseudomonas aeruginosa*-induced mucin overproduction in epithelial cells. Proc Natl Acad Sci USA 1998;95;5718-5723.

38. Li D, Gallup M, Fan N, Szymkowski DE, Basbaum CB. Cloning of the amino-terminal and 5'-flanking region of the human MUC5AC mucin gene and transcriptional up-regulation by bacterial exoproducts. J Biol Chem 1998;273:6812-6820.

39. Levine SJ, Larivee P, Logun C, Angus CW, Ognibene FP, Shelhamer JH. Tumor necrosis factor-alpha induces mucin hypersecretion and *MUC2* gene expression by human airway epithelial cells. Am J Respir Cell Mol Biol 1995;12:196-204.

40. Velcich A, Augenlicht LH. Regulated expression of an intestinal mucin gene in HT29 colonic carcinoma cells. J Biol Chem 1993;268:13956-13961.

41. Jarry A, Merlin D, Velcich A, Hopfer U, Augenlicht LH, Laboisse CL. Interferon-γ

modulates cAMP-induced mucin exocytosis without affecting mucin gene expression in a human colonic goblet cell line. Eur J Pharm 1994;267:95-103.

42. Guzman K, Gray TE, Yoon JH, Nettesheim P. Quantitation of mucin RNA by PCR reveals induction of both MUC2 and MUC5AC mRNA levels by retinoids. Am J Physiol 1996;271:L1023-L1028.

43. Lencer WI, Reinhart FD, Neutra MR. Interaction of cholera toxin with cloned human goblet cells in monolayer culture. Am J Physiol 1990;258:G96-102.

44. McCool DJ, Marcon MA, Forstner JF, Forstner GG. The T84 human colonic adenocarcinoma cell line produces mucin in culture and releases it in response to various secretagogues. Biochem J 1990;267:491-500.

45. Forstner JF, Roomi NW, Fahim REF, Forstner GG. Cholera toxin stimulates secretion of immunoreactive intestinal mucin. Am J Physiol 1981;240:10-16.

46. Chadee K, Keller K, Forstner J, Innes DJ, Ravdin JI. Mucin and nonmucin secretagogue activity of *Entamoeba histolytica* and cholera toxin in rat colon. Gastroenterology 1991;100:986-997.

47. Laburthe M, Augeron C, Rouyer-Fessard C, Roumagnac I, Maoret J, Grasset E, Laboisse C. Functional VIP receptors in the human mucus-secreting colonic epithelial cell line Cl.16E. Am J Physiol 1989;256:G443-450.

48. Seidler U, Knafla K, Kownatzki R, Sewing K. Effect of endogenous and exogenous

prostaglandins on glycoprotein synthesis and secretion in isolated rabbit gastric mucosa. Gastroenterology 1988;95:945-951.

49. Phillips TE, Stanley CM, Wilson J. The effect of 16,16-dimethyl prostaglandin E_2 on proliferation of an intestinal goblet cell line and its synthesis and secretion of mucin glycoproteins. Pros Leuk Essen Fatty Acids 1993;48: 423-428.

50. Moore BA, Sharkey KA, Mantle M. Role of 5-HT in cholera toxin-induced mucin secretion in the rat small intestine. Am J Physiol 1996;270: G1001-G1009.

51. Bou-Hanna C, Berthon B, Combettes L, Claret M, Lamboisse CL. Role of calcium in carbachol- and neurotensin-induced mucin exocytosis in a human colonic goblet cell line and cross-talk with the cyclic AMP pathway. Biochem J 1994;299:579-585.

52. Keller K, Olivier M, Chadee K. The fast release of mucin secretion from human colonic cells induced by *Entameba histolytica* is dependent on contact and protein kinase C activation. Arch Med Res 1992;23:217-221.

53. Seidler U, and Pfeiffer A, 1989. Ca^{2+}-dependent and -independent secretagogue action on gastric mucus secretion in rabbit mucosal explants. Am J Physiol 256: G739-746.

54. Yedgar S, Eidelman O, Malden E, Roberts D, Etcheberrigaray R, Goping G, Fox C, Pollard HB, 1992. Cyclic AMP-independent secretion of mucin by SW1116 human colon carcinoma cells. Biochem J 283: 421-426.

55. Hong D-H, Forstner JF, Forstner G. Protein kinase C-ε is the likely mediator of mucin exocytosis in human colonic cell lines. Am J Physiol 1997;272:G31-G37.

56. Forstner G, Zhang Y, McCool D, Forstner J. Regulation of mucin secretion in T84 adenocarcinoma cells by forskolin: relationship to Ca^{2+} and PKC. Am J Physiol 1994;266:G606-G612.

57. Shimazaki Y, Nishiki T, Omori A, Sekiguchi M, Kamata Y, Kozaki S, Takahashi M. Phosphorylation of 25-kDa synaptosome-associated protein. Possible involvement in protein kinase C-mediated regulation of neurotransmitter release. J Biol Chem 1996;271:14548-14553.

58. Hirling H, Scheller RH. Phosphorylation of synaptic vesicle proteins: modulation of the SNAP interaction with the core complex. Proc Natl Acad Sci USA 1996;93:11945-11949.

59. Bresalier RS, Ho SB, Schoeppner HL, Kim YS, Sleisinger MH, Brodt P, Byrd JC. Enhanced sialylation of mucin-associated carbohydrate structures in human colon cancer metastasis. Gastroenterology 1996;110: 1354-1367.

60. Sternberg LR, Byrd JC, Yunker CK, Dudas S, Hoon VK, Bresalier RS. Liver colonization by human colon cancer cells is reduced by antisense inhibition of MUC2 mucin synthesis. Gastroenterology 1999;116:363-371.

61. Ho SB, Niehans GA, Lyftogt C, Yan PS, Cherwitz DL, Gum ET, Dahiya R, Kim YS.

Heterogeneity of mucin gene expression in normal and neoplastic tissues. Cancer Res 1993;53:641-651.

62. Pullan RD, Thomas GA, Rhodes M, Newcombe RG, Williams GT, Allen A, Rhodes J. Thickness of adherent mucus gel on colonic mucosa in humans and its relevance to colitis. Gut 1994;35:353-359.

63. Schultsz C, Van Den Berg FM, Ten Kate FW, Tytgat GNJ, Dankert J. The intestinal mucus layer from patients with inflammatory bowel disease harbors high numbers of bacteria compared with controls. Gastroenterology 1999;117:1089-1097.

64. Van Klinken BJW, van der Wal JWG, Einerhand AWC, Buller HA, Dekker J. Sulphation and secretion of the predominant secretory human colonic mucin MUC2 in ulcerative colitis. Gut 1999;44:387-393.

65. Tytgat KMAJ, van der Wal JWG, Einerhand AWC, Buller HA, Dekker J. Quantitative analysis of MUC2 synthesis in ulcerative colitis. Biochem Biophys Res Commun 1996;224:397-405.

66. Tytgat KMAJ, Dekker J, Buller HA. Mucins in inflammatory bowel disease. Eur J Gastroenterol Hepatol 1993;5:119-128.

67. Yolken RH, Willoughby R, Wee SB, Miskuff R, Vonderfecht S. Sialic acid glycoproteins inhibit *in vitro* and *in vivo* replication of rotaviruses. J Clin Invest 1987;79:148-154.40.

68. McSweegan E, Walker RI. Identification and characterization of two *Campylobacter jejuni* adhesins for cellular and mucous substrates. Infect Immun 1986;53:141-148.

69. Sajjan SU, Forstner JF. Role of the putative ``link'' glycopeptide of intestinal mucins in binding of piliated *Escherichia coli* serotype O157:H7 strain CL-49. Infect Immun 1990;58:868-873.

70. Sajjan SU, Forstner JF. Characteristics of binding of *Escherichia coli* serotype O157:H7 strain CL-49 to purified intestinal mucin. Infect Immun 1990;58:860-867.

71. Metcalfe JW, Krogfelt KA, Krivan HC, Cohen PS, Laux DC. Characterization and identification of a porcine small intestine mucus receptor for the K88ab fimbrial adhesin. Infect Immun 1991;59:91-96.

72. Mouricout MA, Julien RA. Pilus-mediated binding of bovine enterotoxigenic *Escherichia coli* to calf intestinal mucins. Infect Immun 1987;55:1216-1223.

73. Ensgraber M, Loos M. A 66-kilodalton heat shock protein of *Salmonella typhimurium* is responsible for binding of the bacterium to intestinal mucus. Infect Immun 1992;60:3072-3078.

74. Rajkumar R, Devaraj H, Niranjali S. Binding of *Shigella* to rat and human intestinal mucin. Mol Cell Biochem 1998;178:261-268.

75. Yamamoto T, Yokota T. Electron microscopic study of *Vibrio cholerae* O1 adherence to the mucus coat and villus surface in the human small intestine. Infect Immun

1988;56:2753-2759.

76. Mantle M, Husar SD. Adhesion of *Yersinia enterocolitica* to purified rabbit and human intestinal mucin. Infect Immun 1993;61:2340-2346.

77. Mantle M, Husar SD. Binding of *Yersinia enterocolitica* to purified, native small intestinal mucins from rabbits and humans involves interactions with the mucin carbohydrate moiety. Infect Immun 1994;62:1219-1227.

78. Chadee K, Petri Jr. WA, Innes DJ, Ravdin JI. Rat and human colonic mucins bind to and inhibit the adherence lectin of *Entamoeba histolytica*. J Clin Invest 1987;80:1245-1254.

Chapter 3. Prostaglandin E_2 (PGE_2): Biosynthesis and mode of action[1]

3.1 Arachidonic Acid

Prostaglandins and thromboxanes, collectively termed prostanoids, are produced from the C_{20} polyunsaturated arachidonic acid which is abundant in cellular membranes of mammalian cells where it is esterified into glycerophospholipids. Hormonal and inflammatory stimuli activate phospholipase enzymes which release arachidonate from the *sn*-2 position of membrane phospholipids through hydrolysis of the ester linkage (1). Phospholipase A_2 (PLA_2) liberates arachidonate from phosphatidylethanolamine and phosphatidylcholine whereas phospholipase C combined with diacylglycerol lipase act on phosphatidylinositol derivatives: the predominant source of free intracellular arachidonate is generated by the action of PLA_2 (1). Once released, arachidonate can be oxygenated by two isoforms of the enzyme cyclooxygenase (COX, prostaglandin H synthase; 8,11,14,-icosatrienoate hydrogen donor: oxygen oxidoreductase, EC 1.14.99.1) to form prostaglandins and thromboxanes (Figure 1).

3.2 COX-1 and COX-2

One of the rate-limiting steps in the production of prostanoids is conversion of arachidonic acid to prostanoids by COX. The COX isoform first identified was the

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[1]Portions of this Chapter were adapted from the following reference:

Belley A, Chadee K. Eicosanoid production by parasites: From pathogenesis to immunomodulation ? Parasitol Today 1995;11:327-334.

constitutively expressed enzyme termed COX-1. COX-1 is a glycoprotein complex of two identical heme-containing subunits of 70 kDa and is expressed by virtually all cell types

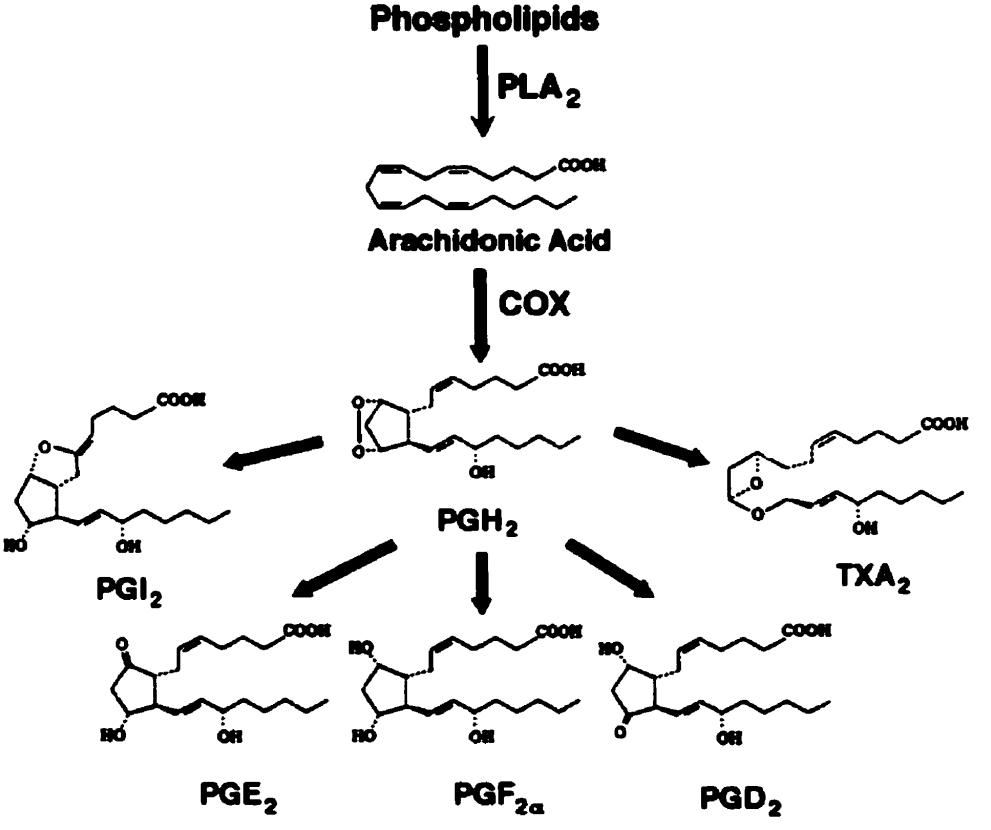

Figure 1. The prostanoid pathway

under normal physiologic conditions (2, 3). Its amino acid identity is highly conserved amongst mammalian species (>80%). COX-1 is a monotopic membrane protein present in the endoplasmic reticulum and nucleus of mammalian cells (4, 5). Crystallographic studies revealed that the protein contains three distinct domains: a membrane binding domain, an epidermal growth factor-like domain and a catalytic region (4). Targeting of the nascent

protein to the endoplasmic reticulum is mediated by a KDEL-like retention signal at the C-terminus. The catalytic region of COX-1 contains a cyclooxygenase and a peroxidase activity which are physically and functionally separate (6). The cyclooxygenase activity is contained within a hydrophobic channel and is mediated by a tyrosine present at position 385. Initiation of the cyclooxygenase activity is interesting in that it requires a hydroperoxide to start the reaction. It has been proposed that oxidation of the heme prosthetic group at the peroxidase active site by an alkyl peroxide or peroxynitrite then oxidizes tyrosine-385 to form a tyrosyl radical which initiates the *bis*cyclo-oxygenation of arachidonate by abstracting the (13S)-hydrogen. Following cyclization and oxygen addition at C-15 to yield PGG_2, the intermediate is then reduced by the peroxidase activity to form PGH_2. Conversion of PGH_2 to the active prostanoid metabolites is mediated by endoperoxide-D isomerase, endoperoxide-E isomerase and endoperoxide reductase which produce PGD_2, PGE_2 and $PGF_{2\alpha}$, respectively (7). Thromboxanes and prostacyclins are formed when PGH_2 is enzymatically converted by thromboxane and prostacyclin synthetases, respectively (7).

A second COX isoform, termed COX-2, was initially identified in chicken and mouse fibroblasts in 1991 (8, 9). It converts arachidonic acid to prostaglandins similar to COX-1 and is highly conserved in mammals. COX-2 is glycosylated and migrates as a protein doublet with a molecular mass of 70-74 kDa by SDS-PAGE (10-13). *N*-linked glycosylation is required for maximum activity of both isoforms (14). There is 61% identity between the deduced hCOX-1 and hCOX-2 amino acid sequences (13). The catalytic mechanism of COX-2 is essentially identical to COX-1 however the tyrosine residue is at position 371. The major difference between the two isoforms is their pattern of expression. COX-2 mRNA and protein is usually only detected after stimulation with

various agents such as interleukin-1 (IL-1) (15), TNF-α, PMA (16), serum (17), platelet-derived growth factor (PDGF) (17), lipopolysaccharide (LPS) (18), and PGE_2 (19).

The human COX-1 gene is located on chromosome 9 and is encoded by a 22 kb gene (20). The gene encodes mRNAs of 3 and 5 kb (13). There is no apparent TATA box within the 5'-promoter, a characteristic of housekeeping genes (21). Although the promoter region contains responsive elements such as Sp-1, PEA-3, AP2, NF-IL6 and GATA-1, its expression is virtually unchanged by exogenous stimuli (22). The COX-2 gene spans 8.3 kB, is located on chromosome 1 and encodes an mRNA of 4.5 kB (22, 23). It is considered to be an imediate early gene and its promoter contains a TATA box and several inducible elements such as nuclear factor-κB (NF-κB), CCAAT/enhancer binding protein (CEBP), IL-6 response element, cyclic AMP responsive element (CRE), AP-2, Sp-1, PEA, GATA-1 and glutocorticoid responsive element (24). Furthermore the expression of COX-2 is regulated post-transcriptionally (25).

3.3 Non-steriodal antiinflammatory drugs

In the 1970's, it was discovered that the analgesic and antiinflammatory properties of aspirin were due to inhibition of prostanoid production via inhibition of cyclooxygenase activity (26, 27). Aspirin is one of a group of drugs known as the non-steriodal antiinflammatory drugs (NSAIDs) which include other drugs such as indomethacin and ibuprofen. These drugs sterically hinder binding of arachidonic acid in the active site channel and thus are competitive reversible inhibitors (28). Aspirin, however, covalently modifies the enzyme by acetylating $serine^{529}$ of COX-1 and $serine^{516}$ of COX-2, residues which are highly conserved in the C terminus of all mammalian COX polypeptides (13, 20).

Complications occur in patients undergoing NSAID therapy for inflammatory diseases such as rheumatoid arthritis. The main side-effect in patients is increased risk of bleeding associated with ulcers in the stomach and small intestine (29). However, other side-effects include relapse of inflammatory bowel disease, complications of diverticular disease, non-specific colitis and intestinal strictures. Ulceration may be due to the "non-selectivity" of the current NSAIDs used: these drugs inhibit prostanoid production by both isoforms (30). The high levels of prostanoids produced from the induction of COX-2 by inflammatory stimuli are thought to contribute to and exacerbate the inflammatory response. Because COX-1 is constitutively expressed, its products are thought to maintain tissue homeostasis and therefore abrogation of its function is undesirable. A major effort has been directed towards developing COX-2 specific NSAIDs. It is thought that preferential inhibition of COX-2 without affecting the constitutive production of prostanoids from COX-1 may prevent the deleterious effects associated with prolonged NSAID therapy. Clinical trials with the selective COX-2 inhibitors rofecoxib and celecoxib showed that these drugs were analgesic and did not cause gastroduodenal lesions (31). However, other studies contradicted the hypothesis that COX-2 inhibitors would spare gastrointestinal tissues. The NSAID diclofenac increased intestinal permeability and bacterial load in rat small intestine (32). Diclofenac underwent entero-hepatic recirculation, which increased its ulcerogenic effect on the intestinal mucosa. In contrast, the diclofenac derivative nitrofenac, although increased intestinal permeability, did not increase bacterial load nor cause ulceration. Nitrofenac did not undergo entero-hepatic recirculation. Similarly, the NSAIDs aspirin and nabumetone did not undergo entero-hepatic recirculation nor cause small intestinal damage in this study. In another study, treatment of rats with high doses of the selective COX-1 inhibitor SC-560 did not cause ulcers (33). Thus, the former study showed that NSAID-induced enteropathy is associated with the drug's ability to undergo

entero-hepatic reciculation and act as a topical irritant and not by inhibiting prostaglandin synthesis.

3.4 Role of COX and PGE_2 in Inflammation

Acute and chronic inflammatory responses are typically characterized by increased production of prostanoids produced by tissue and infiltrating cells. PGE_2 is considered a proinflammatory agent: it causes vasodilation of arterioles in the microcirculation which consequently leads to erythema and enhances histamine or bradykinin induced edema (7, 34, 35). Moreover, PGE_2 causes fever and enhances hyperalgesia (36, 37). Studies showed increased expression of COX-2 in the spinal cord after peripheral stimulation and direct administration of NSAIDs to the spinal cord alleviated hyperalgesia, suggesting a role of central nervous system-derived prostaglandins in some pain responses (38-40). Several studies addressed whether COX-1 and/or COX-2 derived prostanoids mediate local edema and hyperalgesia. A pharmacologic analysis of COX in carrageenan-induced inflammation of the rat footpad revealed that although there was increased production of PGE_2, which was inhibited with the highly-specific COX-1 inhibitor SC-560, the NSAID did not reduce edema nor decrease hyperalgesia (41). In contrast, the highly-specific COX-2 inhibitor celecoxib decreased edema and alleviated the hyperalgesic response. Injection of carrageenan into the footpad caused elevated levels of PGE_2 in the cerebrospinal fluid which were inhibitable with celecoxib but not with the COX-1 inhibitor SC-560. Interestingly, in COX-1 deficient mice, there was reduced inflammation induced by topical administration of arachidonic acid in an ear swelling assay after 2 hours (42). The authors suggested that COX-1 derived prostanoids contribute to the inflammatory process, at least in mouse ear tissue. Moreover, arachidonic acid, tetradecanoyl phorbol

acetate and carrageenan induced similar inflammatory responses (ear swelling, paw edema) in COX-2 knockout mice as compared to wild-type mice (43, 44). Thus, studies with COX deficient mice demonstrated that it is not as simple to suggest that COX-2 derived prostanoids mediate inflammation whereas COX-1 derived prostanoids do not.

3.5 Immunomodulation by PGE_2

PGE_2 is generally viewed as an immunosuppressive agent, downregulating numerous B cell, T cell and macrophage functions (45). PGE_2 derived from macrophages, tissue cells, endothelial cells and neutrophils may modulate immune cell functions.

B cells

Regulation of antibody production by B cells may have a direct outcome on disease severity. PGE_2 inhibited antigen and IL-2 induced B-cell proliferation similar to forskolin through a cAMP-dependent mechanism (46). Moreover, it inhibited IgM production and synergized with IL-4 to promote immunoglobulin class-switching to IgE and IgG1 by LPS-stimulated murine B cells (47, 48). It is possible that PGE_2 may indirectly modulate B cell functions through regulation of cytokine production by T cells (49).

T cells

One of the main immunomodulatory effects of PGE_2 is through regulation of cytokine production by $CD4^+$ T cells. PGE_2 inhibited antigen- and mitogen- induced T-cell proliferation (50, 51), interleukin-2 (IL-2) production (50, 52) and IL-2 receptor expression (53) by increasing $[cAMP]_i$. Moreover, it inhibited interferon-γ (IFN-γ) production and IL-1 induced granulocyte-macrophage colony stimulating factor (49, 54). Whereas Th1-cell subset production of lymphokines (IL-2 and IFN-γ) are inhibited by

PGE_2, the Th2 lymphokine IL-4 is not and IL-5 production is slightly increased (49). Furthermore, monocyte production of IL-12 (and IL-12 receptor expression), which promotes differentiation to antigen-specific Th1 lymphocytes, is inhibited by PGE_2 (55, 56). Thus it would appear that PGE_2 tips the balance in favor of a Th2-type cytokine response. Once activated, T cells migrate to inflammatory sites to exert their effects. Transendothelial migration of T cells was shown to be inhibited by treatment of T cells and/or endothelial cells with PGE_2 (57). This response was not caused by alteration of adhesion molecule expression by either cell type. The inhibition of transendothelial migration coincided with increased $[cAMP]_i$.

Macrophages

PGE_2 has diverse effects on accessory and effector cell funtions of macrophages. Exogenous PGE_2 suppresses macrophage expression of the major histocompatibility complex Ia molecule (58) which is ultimately required for effective presentation of antigen to T cells. Moreover, it inhibits macrophage proliferation (59), oxygen radical generation (60), production of IL-1 (61) and LPS-induced TNF-α mRNA accumulation (62, 63).

3.6 PGE_2 receptors

PGE_2 exerts its diverse biological activity by activating four guanine nucleotide binding (GTP or G) protein-linked receptors, termed EP receptors, which are coupled to different intracellular signaling pathways (64). EP receptors are typical G protein coupled receptors (GPCR) with seven transmembrane domains. The G protein consists of a trimer of α, ß and γ subunits. In an inactive state, the G_α subunit is bound to GDP which causes

it to associate with $G_{\beta}G_{\gamma}$. Upon receptor activation, the G_{α} subunit exchanges its GDP for GTP and dislocates from $G_{\beta}G_{\gamma}$. The G_{α} subunit then activates second signaling pathways. Slow hydrolysis of the GTP back to GDP by the inherent GTPase activity of the G_{α} subunit causes it to reassociate with $G_{\beta}G_{\gamma}$ and resume the inactive receptor bound conformation. There are several different forms of the G_{α} subunit and this dictates which second signaling pathway will be activated. For example, the EP_1 receptor is linked to $G_{\alpha o}$ which activates a Ca^{2+} channel to cause an influx of extracellular Ca^{2+} (65) (Figure 2). The EP_2 and EP_4 receptors are both linked to $G_{\alpha s}$ which activates adenylate cyclase to increase $[cAMP]_i$ whereas the EP_3 receptor is linked to $G_{\alpha i}$ which inhibits adenylate cyclase and decreases $[cAMP]_i$ (66-70). The EP_3 receptor may also mediate increases in $[Ca^{2+}]_i$ (71).

The EP_1, EP_3 and EP_4 receptors are not only present in the plasma membrane but are also expressed in the nuclear envelope (72, 73), similar to the nuclear receptor peroxisome proliferator-activated receptor-γ (PPAR-γ) which is activated by the PGD_2 metabolite 15-deoxy-Δ (12,14)-PGJ_2 (74). Thus PGE_2 and other prostanglandins mediate nuclear signaling. However, prostanoids are not stored and are secreted following production. It is unknown whether newly synthesized prostaglandins interact immediately with nuclear receptors or whether they must traverse the plasma membrane and translocate to the nucleus. A prostaglandin tranporter was cloned from a human kidney cDNA library

and may provide the means for extracellular prostaglandins to interact with nuclear EP receptors (75).

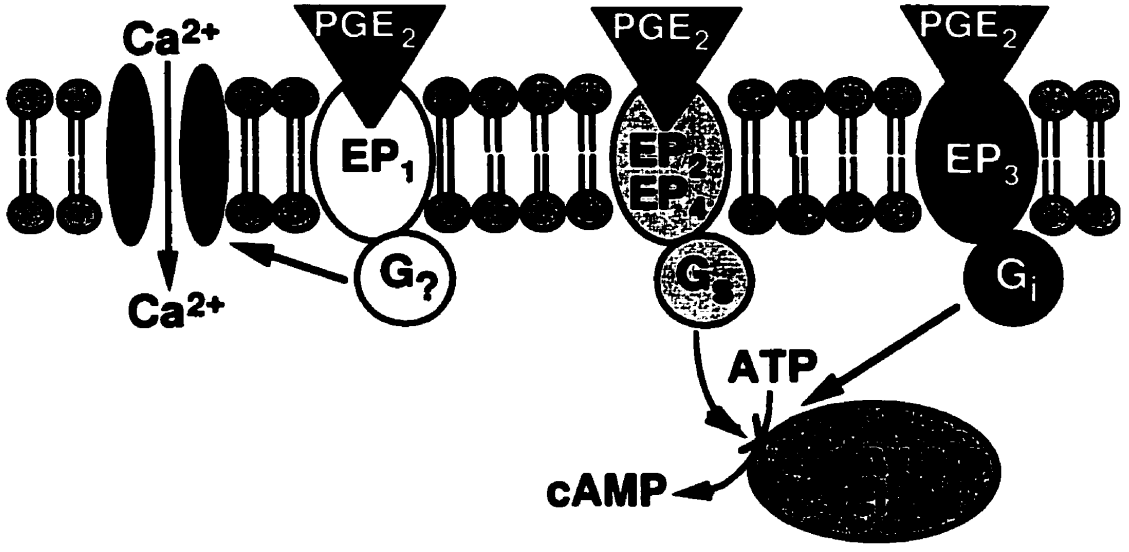

Figure 2. Signaling pathways of PGE_2 receptors

3.7 Role of COX, PGE_2 and EP receptors in gastrointestinal tissues

In the gastrointestinal tract, exogenously added prostaglandins inhibited gastric acid secretion, stimulated intestinal motility, and caused fluid and electrolyte secretion (76). PGE_2 protected the gastric mucosa from noxious agents and reduced the severity of ulceration and inflammation in animal models of colitis (77-79). Immunization of rabbits with PGE_2 caused ulceration in the stomach and small intestine (80). Thus PGE_2 was cytoprotective to gastrointestinal tissues. The cytoprotective nature of PGE_2 may encompass several mechanisms within the gastrointestinal tract. The EP_3 agonist/EP_1

antagonist ONO-NT-012 opened a gastric parietal cell housekeeping Cl^- channel via increases in $[Ca^{2+}]_i$ (71). The integrity of tight junctions is vital to maintaining the epithelial barrier function of the intestinal mucosa. In porcine ileum, PGE_2 and PGI_2 (prostacyclin) synergistically restored tight junction closure between ileal epithelial cells following ischemic injury (81). Furthermore, treatment of ileal mucosa with indomethacin prolonged the recovery of epithelial resistance following ischemia. The response to PGE_2 and PGI_2 was mediated by increases in $[cAMP]_i$ and $[Ca^{2+}]_i$, respectively, which regulate actin fibers of the tight junction. Finally, activation of the EP_4 receptor stimulated mucin exocytosis, another vital component of the epithelial barrier (82).

A new study showed exacerbation of colonic injury in COX-1 and COX-2 deficient mice fed dextran sodium sulfate (DSS) to induce acute inflammation in the colon (83). COX-2 deficient mice were more susceptible to colonic injury than COX-1 mice and treatment of COX-1 deficient mice with the specific COX-2 inhibitor NS-398 exacerbated colonic damage. A specific COX-2 inhibitor also exacerbated colonic injury in rats with preexisting inflammation induced by instillation of trinitrobenzene sulfonic acid (84). These results suggested that either elevated prostaglandins produced by COX-2 are involved in wound healing or that suppression of COX-2 increases inflammation-associated colonic injury.

The role of COX in intestinal diseases has begun to be investigated. Epidemiologic evidence showed that there was a lower risk of colon cancer in humans using NSAIDs continuously (85). Expression of COX-2 protein was detected in colon cancer tissue whereas there was no expression in nontumorous tissue (86). Increased prostaglandin production as a result of increased COX-2 expression stimulated proliferation and

prevented apoptosis of colonocytes (87, 88). Ablation of the COX-2 gene or inhibition of enzyme activity with a COX-2 inhibitor decreased the number and size of polyps in the $APC^{\Delta 716}$ mouse model of familial adenomatous polyposis (89). In normal, Crohn's disease, and ulcerative colitis colonic epithelium, there was no change of expression of COX-1, which was localized to colonic crypt cells (90). No expression of COX-2 was observed in nomal colonic mucosa. In contrast, COX-2 was highly expressed in apical epithelial cells and lamina propria mononuclear cells in Crohn's disease and ulcerative colitis. Increased epithelial PGE_2 may contribute to the diarrhea and cell proliferation associated with inflammatory bowel disease (90).

3.8 Prostanoid production by parasites

To date, COX-1 and COX-2 enzymes have only been described in vertebrates and phylogenetically the diversity of the enzymes in invertebrates is unknown. Many parasites species have been shown to produce PGE_2 and other prostanoids, as summarized in Table 1. The predominant hypothesis is that parasite-derived prostanoids stifle the host response, inhibiting macrophage and T and B cell functions. Parasite-derived PGE_2 could modulate the immune response and affect the severity of infection: PGE_2 promotes a Th2 cytokine profile, which in certain parasitic infections (schistosome eggs), does not offer protection (102). It is also possible that parasite-derived prostanoids are involved in pathogenesis and pathophysiology. For example, dogs experience respiratory difficulties and reduced exercise tolerance when infected with the canine heartworm, *Dirofilaria immitis*. Treatment of heartworms with indomethacin abrogated filarial-induced depression of rat aorta whereas pretreatment of rat thoracic aorta vascular rings with aspirin or indomethacin did not (101). PGD_2 was detected in chloroform extracts of *D. immitis* bioassays by GC-MS but not in

control or aspirin-treated worm bioassays. These results may explain the reduced exercise tolerance in dogs infected with *D. immitis* and implicate heartworm-derived PGD_2 in the pathogenesis of canine heartworm disease. PGD_2, a somnogenic substance in humans,

Table 1. Production of prostanoids by various parasites

Parasite	Prostanoid	Method[a]	Reference
Protozoa			
Acanthamoeba castellanii	PGE_2,	TLC	90
Plasmodium falciparum	PGD_2, PGE_2, $PGF_{2\alpha}$	EIA, GC-MS	91
Trematodes			
Schistosoma mansoni	PGE_2, PGE_1	RIA, HPLC	92, 93
Trichobiharzia ocellata	PGE_1	RIA, HPLC	93
Cestodes			
Taenia taeniaeformis	PGE_2, PGI_2, TXA_2	RIA, TLC	94, 95
Spirometra erinacei	PGE_2	GC-MS	96
Nematodes			
Brugia malayi	PGD_2, PGE_2, PGI_2	RIA, TLC, HPLC	97, 98
Wuchereria bancrofti	PGE_2	IFL	98
Dirofilaria immitis	PGD_2, PGE_2, PGI_2	RIA, HPLC, GC-MS	99, 100

[a]Method of identification of prostanoids: EIA, enzyme immunoassay; GC-MS, gas chromatography-mass spectometry; HPLC, high-performance liquid chromatography; IFL, immunofluorescent localization; RIA, radioimmunoassay, TLC, thin-layer chromatography

was produced by *Plasmodium falciparum* and may contribute to sleepiness observed in patients with acute malarial infections (92). Microfilaria of *Brugia malayi* circulate in the

blood and reside in the lymphatics. The parasite produced PGE_2 and PGI_2, which may dilate blood vessels and prevent adherence of platelets, respectively (98).

Logically, parasite-derived eicosanoids may act in an autocrine manner, regulating parasite development or homeostatic functions. Prostaglandin production by *S. mansoni* cercariae was correlated with cercarial transformation (103). However, another study contradicted this finding and showed that cyclooygenase inhibitors did not alter the ability of linoleic acid to stimulate cercarial tail loss (104). Moreover, *trans*-isomers of fatty acids are not converted to prostanoids but can induce cercarial tail loss.

3.9 References

1. Smith WL, Marnett, LJ. Prostaglandin endoperoxide synthase: structure and catalysis. Biochim Biophys Acta 1991;1083;1-17.

2. Boyd J. Prostaglandin H synthase. Meth Enzymol 1990;186:283-287.

3. Wu KK. Regulation of prostaglandin H synthase-1 gene expression. Adv Exp Med Biol 1997;400A:167-170.

4. Picot D, Loll PJ, Garavito, RM. The X-ray crystal structure of the membrane protein prostaglandin H_2 synthase-1. Nature 1994;367:243-249.

5. Spencer AG, Woods JW, Arakawa T, Singer II, Smith WL. Subcellular localization of prostaglandin endoperoxide H synthases-1 and -2 by immunoelectron microscopy. J Biol Chem 1998;273:9886-9893.

6. Smith WL, Garavito RM, DeWitt DL. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J Biol Chem 1996;271:33157-33160.

7. Williams KI, Higgs GA. Eicosanoids and inflammation. J Pathol 1988;156:101-110.

8. Kujubu DA, Fletcher BS, Varnum BC, Lim RW, Herschman HR. TIS10, a phorbol ester tumor promoter-inducible mRNA from swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. J Biol Chem 1991;266:12866-12872.

9. Xie W, Robertson DL, Simmons DL. Mitogen-inducible prostaglandin G/H synthase: A new target for nonsteroidal anti-inflammatory drugs. Drug Dev Res 1992;25:249-265.

10. Habib A, Creminon C, Frobert Y, Grassi J, Pradelles P, Maclouf J. Demonstration of an inducible cyclooxygenase in human endothelial cells using antibodies raised against the carboxyl-terminus region of the cyclooxygenase-2. J Biol Chem 1993;268:23448-23454.

11. Holtzman MJ, Turk J, Shornick LP. Identification of a pharmacologically distinct prostaglandin H synthase in cultured epithelial cells. J Biol Chem 1992;267:21438-21445.

12. Pritchard KA, O'Banion MK, Miano JM, Vlasic N, Bhatia UG, Young DA, Stemerman MR. Induction of cycloxygenase-2 in rat vascular smooth muscle cells *in vitro* and *in vivo*. J Biol Chem 1993;269:8504-8509.

13. Hla T, Nielson K. Human cyclooxygenase-2 cDNA. Proc Natl Acad Sci USA 1992;89:7384-7388.

14. O'Neill GP, Mancini JA, Kargman S, Yergey J, Kwan MY, Falgueyret JP, Abramovitz M, Kennedy BP, Ouellet M, Cromlish W, Culp S, Evans JF, Ford-Hutchinson AW, Vickers PJ. Overexpression of human prostaglandin G/H synthase-1 and -2 by recombinant vaccinia virus: inhibition by nonsteroidal anti-inflammatory drugs and biosynthesis of 15-hydroxyeicosatetraenoic acid. Mol Pharmacol 1993;45:245-254.

15. Raz A, Wyche A, Siegel N, Needleman P. Regulation of fibroblast cyclooxygenase synthesis by interleukin 1. J Biol Chem 1988;263:3022-3028.

16. Raz A, Wyche A., Needleman P. Temporal and pharmacological division of fibroblast cyclooxygenase expression into transcriptional and translational phases. Proc Natl Acad Sci USA 1989;86:1657-1661.

17. Lin AH, Bienkowski MJ, Gorman RR. Regulation of prostaglandin H synthase mRNA levels and prostaglandin biosynthesis by platelet-derived growth factor. J Biol Chem 1989;264:17379-17383.

18. Bienkowski MJ, Petro MA, Robinson LJ. Inhibition of thromboxane A_2 synthesis in U937 cells by glucocorticoids. J Biol Chem 1989;264:6536-6544.

19. Takahashi Y, Taketani Y, Endo T, Yamamoto S, Kumegawa M. Studies on the induction of cyclooxygenase isozymes by various prostaglandins in a mouse osteoblastic cell line with reference to signal transduction pathways. Biochim Biophys Acta 1994;1212:217-224.

20. Funk CD, Funk LB, Kennedy ME, Pong AS, Fitzgerald GA. Human platelet/erythroleukemia cell prostaglandin G/H synthase: cDNA cloning, expression and gene chromosomal assignment. FASEB J 1991;5:2304-2312.

21. Kraemer SA, Meade SA, DeWitt DL. Prostaglandin endoperoxide synthase gene structure: identification of the transcriptional start site and 5'-flanking regulatory sequences. Arch Biochem Biophys 1992;293:391-400.

22. Wu KK. Inducible cyclooxygenase and nitric oxide synthase. Adv Pharmacol 1995;33:179-207.

23. Fletcher BS, Kujubu DA, Perrin DM, Herschman HR. Structure of the mitogen-inducible TIS10 gene and demonstration that the TIS10-encoded protein is a functional prostaglandin G/H synthase. J Biol Chem 1992;267:4338-4344.

24. Appleby SB, Ristimaki A, Neilson K, Narko K, Hla T. Structure of the cyclooxygenase-2 gene. Biochem J 1994;302:723-727.

25. Ristimaki A, Garfinkel S, Wessendorf J, Maciag T, Hla T. Induction of cyclooxygenase-2 by interleukin-1 alpha. Evidence for post-transcriptional regulation. J Biol Chem 1994;269:11769-11675.

26. Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspririn-like drugs. Nature New Biol 1971;231:232-235.

27. Smith JB, Willis AL. Aspirin selectively inhibits prostaglandin production in human platelets. Nature New Biol 1971;231:235-239.

28. Smith WL, DeWitt DL. Prostaglandin endoperoxide H synthases-1 and -2. Adv Immunol 1996;62:167-215.

29. Bjarnason I, Hayllar J, MacPherson AJ, Russell AS. Side effects of nonsteroidal anti-inflammatory drugs on the small and large intestine in humans. Gastroenterology 1993;104:1832-1847.

30. Vane J. Towards a better aspirin. Nature 1994;367:215-216.

31. Laine L, Harper S, Simon T, Bath R, Johanson J, Schwartz H, Stern S, Quan H, Bolognese J. A randomized trial comparing the effect of rofecoxib, a cyclooxygenase 2-specific inhibitor, with that of ibuprofen on the gastroduodenal mucosa of patients with osteoarthritis. Gastroenterology 1999;117:776-783.

32. Reuter BK, Davies NM, Wallace JL. Nonsteroidal anti-inflammatory drug enteropathy in rats: role of permeability, bacteria, and enterohepatic circulation. Gastroenterology 1997;112:109-117.

33. Palmer RH. COX-2 selectivity and lack of gastrointestinal toxicity: True, true and unrelated? Gastroenterology 2000;118;640-641.

34. Williams TJ, Morley J. Prostaglandins as potentiatiors of increased vascular permeability in inflammation. Nature 1973;246:215-217.

35. Moncada S, Ferreira SH, Vane JR. Prostaglandins, aspirin-like drugs and the oedema of inflammation. Nature 1973;246:217-219.

36. Ushikubi F, Segi E, Sugimoto Y, Murata T, Matsuoka T, Kobayashi T, Hizaki H, Tuboi K, Katsuyama M, Ichikawa A, Tanaka T, Yoshida N, Narumiya S. Impaired febrile response in mice lacking the prostaglandin E receptor subtype EP_3. Nature 1998;395:281-284.

37. Seibert K, Zhang Y, Leahy K, Hauser S, Masferrer J, Perkins W, Lee L, Isakson PC Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. Proc Natl Acad Sci USA 1994;91:12013-12017.

38. Malmberg AB, Yaksh TL. Hyperalgesia mediated by spinal glutamate or substance P receptor blocked by spinal cyclooxygenase inhibition. Science 1992;257:1276-1279.

39. Malmberg AB, Yaksh TL. Antinociceptive actions of spinal nonsteroidal anti-inflammatory agents on the formalin test in the rat. J Pharmacol Exp Ther 1992;263:136-146.

40. Beiche F, Scheuerer S, Brune K, Geisslinger G, Goppelt-Struebe M. Up-regulation of cyclooxygenase-2 mRNA in the rat spinal cord following peripheral inflammation. FEBS Lett 1996;390:165-169.

41. Smith CJ, Zhang Y, Koboldt CM, Muhammad J, Zweifel BS, Shaffer A, Talley JT, Mansferrer JL, Seibert K, Isakson PC. Pharmacological analysis of cyclooxygenase-1 in inflammation. Proc Natl Acad Sci USA 1998;95:13313-13318.

42. Langenbach R, Morham SG, Tiano HF, Loftin CD, Ghanayem BI, Chulada PC, Mahier JF, Lee CA, Goulding EH, Kluckman KD, Kim HS, Smithies O. Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. Cell 1995;83:483-492.

43. Morham SG, Langenbach R, Loftin CD, Tiano HF, Vouloumanos N, Jennette JC, Mahier JF, Kluckman KD, Ledford A, Lee CA, Smithies O. Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. Cell 1995;83:473-482.

44. Dinchuk JE, Car BD, Focht RJ, Johnston JJ, Jaffee BD, Covington MB, Contel NR, Eng VM, Collins RJ, Czerniak PM, Gorry SA, Trzaskos JM. Renal abnormalities and an altered inflammatory response in mice lacking cyclooxygenase II. Nature 1995;378:406-409.

45. Goodwin JS, Cueppens J. Regulation of the immune response by prostaglandins. J Clin Immunol 1983;3:295-315.

46. Simkin NJ, Jelinek DF, Lipsky PE. Inhibition of human B cell responsiveness by prostaglandin E_2. J Immunol 1987;138:1074-1081.

47. Roper RL, Conrad DH, Brown DM, Warner GL, Phipps RP. Prostaglandin E_2 promotes IL-4 induced IgE and IgG1 synthesis. J Immunol 1990;145:2644-2651.

48. Roper RL, Phipps RP. Prostaglandin E_2 and cAMP inhibit B lymphocyte activation and simultaneously promote IgE and IgG1 synthesis. J Immunol 1992;149:2984-2991.

49. Betz M, Fox BS. Prostaglandin E_2 inhibits production of Th1 lymphokines but not of Th2 lymphokines J Immunol 1991;146:108-113.

50. Minakuchi R, Wacholtz MC, Davis LS, Lipsky PE. Delineation of the mechanism of inhibition of human T cell activation by PGE_2. J Immunol 1990;145:2616-2625.

51. Ellner JJ, Spagnuolo PJ. Suppression of antigen- and mitogen-induced human T-lymphocyte DNA synthesis by bacterial lipopolysaccharide: mediation by monocyte activation and production of prostaglandins. J. Immunol. 1979;123:2689-2695.

52. Rappaport RS, Dodge GR. Prostaglandin E inhibits the production of human interleukin 2. J Exp Med 1982;155:943-948.

53. Rincon M, Tugores A, Lopez-Rivas A, Silva A, Alonso M, Delandazuri MO, Lopez-Botet M. Prostaglandin E_2 and the increase in intracellular cAMP inhibit the expression of interleukin 2 receptors in human T cells. Eur J Immunol 1988;18:1791-1796.

54. Borger P, Kauffman HF, Vijgen JL, Postma DS, Vellenga E. Activation of the cAMP-dependent signaling pathway downregulates the expression of interleukin-3 and granulocyte-macrophage colony-stimulating factor in activated human T lymphocytes. Exp Hematology 1996;24:108-115.

55. Monteleone G, Parrello T, Monteleone I, Tammaro S, Luzza F, Pallone F. Interferon-γ (IFN-γ) and prostaglandin E_2 (PGE_2) regulate differently IL-12 production in human intestinal lamina propria mononuclear cells (LPMC). Clin Exp Immunol 1999;117:469-75.

56. Wu CY, Wang K, McDyer JF, Seder RA. Prostaglandin E2 and dexamethasone inhibit IL-12 receptor expression and IL-12 responsiveness. J Immunol 1998;161:2723-2730.

57. Oppenheimer-Marks N, Kavanaugh AF, Lipsky PE. Inhibition of the transendothelial migration of human T lymphocytes by prostaglandin E_2. J Immunol 1994;152:5703-5713.

58. Snyder DS, Beller DI, Unanue ER. Prostaglandins modulate macrophage Ia expression. Nature 1982;299:163-165.

59. Pelus LM, Broxmeyer HE, Kurland JL. Regulation of macrophage and granulocyte proliferation. J Exp Med 1982;150:277-292.

60. Smith RL, Weidemann MJ. Reactive oxygen production associated with arachidonic acid metabolism by peritoneal macrophages. Biochem Biophys Res Commun 1980;97:973-980.

61. Kunkel SL, Chensue SW, Phan SH. Prostaglandins as endogenous mediators of interleukin 1 production. J Immunol 1986;136:186-192.

62. Kunkel SL, Spengler M, May MA, Spengler R, Larrick J, Remick D. Prostaglandin E_2 regulates macrophage-derived tumor necrosis factor gene expression. J Biol Chem 1988;263:5380-5384.

63. Kunkel SL, Siggins RC, Chensue SW, Larrick J. Regulation of macrophage tumor necrosis factor production of prostaglandin E_2. Biochem Biophys Res Commun 1986;137:404-410.

64. Negishi M, Sugimoto Y, Ichikawa, A. Prostaglandin E receptors. J Lipid Mediators Cell Signal 1995;12:379-391.

65. Funk C, Furci L, FitzGerald G, Grygorczyk R, Rochette C, Bayne MA, Abramovitz M, Adam M, Metters KM. Cloning and expression of a cDNA for the human prostaglandin E receptor EP_1 subtype. J Biol Chem 1993;268:26767-26772.

66. Regan JW, Bailey TJ, Pepperl DJ, Pierce KL, Bogardus AM, Donello JE, Fairbairn CE, Kedzie KM, Woodward DF, Gil DW. Cloning of a novel human prostaglandin receptor with characteristics of the pharmacologically defined EP_2 subtype. Mol Pharmacol 1994;46:213-220.

67. An S, Yang J, Xia M, Goetzl EJ. Cloning and expression of the EP_2 subtype of human receptors for prostaglandin E_2. Biochem Biophys Res Commun 1993;197:263-270.

68. Bastien L, Sawyer N, Grygorczyk R, Metters KM, Adam M. Cloning, functional expression, and characterization of the human prostaglandin E_2 receptor EP_2 subtype. J Biol Chem 1994;269:11873-11877.

69. Adam M, Boie Y, Rushmore TH, Müller G, Bastien L, McKee KT, Metters KM, Abramovitz M. Cloning and expression of the three isoforms of the human EP_3 prostanoid receptor. FEBS Lett 1994;338:170-174.

70. Regan JW, Bailey TJ, Donello JE, Pierce KL, Pepperl DJ, Zhang D, Kedzie KM, Fairbairn CE, Bogardus AM, Woodward DF, Gil DW. Molecular cloning and expression of human EP_3 receptors: evidence of three variants with differing carboxyl termini. Br J Pharmacol 1994;112:377-385.

71. Sakai H, Kumano E, Ikari A, Takeguchi N. A gastric housekeeping Cl^- channel activated via prostaglandin EP_3 receptor-mediated Ca^{2+}/Nitric oxide/cGMP pathway. J Biol Chem 1995;270:18781-18785.

72. Bhattacharya M, Peri KG, Almazan G, Ribeiro-da-Silva A, Shichi H, Durocher Y, Abramovitz M, Hou X, Varma DR, Chemtob S. Nuclear localization of prostaglandin E_2 receptors. Proc Natl Acad Sci USA 1998;95:15792-15797.

73. Bhattacharya M, Peri K, Ribeiro-da-Silva A, Almazan G, Shichi H, Hou X, Varma DR, Chemtob S. Localization of functional prostaglandin E_2 receptors EP_3 and EP_4 in the nuclear envelope. J Biol Chem 1999;274:15719-15724.

74. Kliewer SA, Lenhard JM, Willson TM, Patel I, Morris DC, Lehmann JM. A prostaglandin J_2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. Cell 1995;83:813-819.

75. Lu R, Kanai N, Bao Y, Schuster VL. Cloning, in vitro expression, and tissue distribution of a human prostaglandin transporter cDNA (hPGT). J Clin Invest 1996;98:1142-1149.

76. Whittle BJR, Vane JR. Prostanoids as regulators of gastrointestinal function. In: Johnson LR, ed. Physiology of the gastrointestinal tract. Volume 2. 2nd ed. New York: Raven, 1987:143-179.

77. Wallace JL, Whittle BJR, Boughton-Smith NK. Prostaglandin protection of rat colonic mucosa from damage induced by ethanol. Dig. Dis. Sci. 1985;30:866-876.

78. Allgayer H, Deschryer K, Stenson WF. Treatment with 16,16-dimethyl prostaglandin E_2 before and after induction of colitis with trinitrobenzenesulfonic acid in rats decreases inflammation. Gastroenterology 1989;96:1290-1300.

79. Fedorak RN, Empey LR, MacArthur C, Jewell LD. Misoprostol provides a colonic mucosal protective effect during acetic acid-induced colitis in rats. Gastroenterology 1990;98:615-625.

80. Redfern JS, Feldman M. Role of endogenous prostaglandins in preventing gastrointestinal ulceration: induction of ulcers by antibodies to prostaglandins. Gastroenterology 1989;96:596-605.

81. Blikslager AT, Roberts MC, Rhoads JM, Argenzio RA. Prostaglandins I_2 and E_2 have a synergistic role in rescuing epithelial barrier function in porcine ileum. J Clin Invest 1997;100:1928-1933.

82. Belley A, Chadee K. Prostaglandin E_2 stimulates rat and human colonic mucin exocytosis via the EP_4 receptor. Gastroenterology 1999;117:1352-1362.

83. Morteau O, Morham SG, Sellon R, Dieleman LA, Langenbach R, Smithies O, Sartor RB. Impaired mucosal defense to acute colonic injury in mice lacking cyclooxygenase-1 or cyclooxygenase-2. J Clin Invest 2000;105:469-478.

84. Reuter BK, Asfaha S, Buret A, Sharkey KA, Wallace JL. Exacerbation of inflammation-associated colonic injury in rat through inhibition of cyclooxygenase-2. J Clin Invest 1996;98:2076-2085.

85. Thun MJ, Namboodiri MM, Heath CWJ. Aspirin use and reduced risk of fatal colon cancer. N Engl J Med 1991;325:1593-1596.

86. Kargman SL, O'Neill GP, Vickers PJ, Evans JF, Mancini JA, Jothy S. Expression of prostaglandin G/H synthase-1 and -2 protein in human colon cancer. Cancer Res 1995;55:2556-2559.

87. Kutchera W, Jones DA, Matsunami N, Groden J, McIntyre TM, Zimmerman GA, White RL, Prescott SM. Prostaglandin H synthase 2 is expressed abnormally in human colon cancer. Proc Natl Acad Sci USA 1996;93:4816-4820.

88. Tsujii M, DuBois RN. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. Cell 1995;83:493-501.

89. Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hanconck B, Kwong E, Trzaskos JM, Evans JF, Taketo MM. Suppression of intestinal polyposis in $APC^{\Delta 716}$ knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell 1996;87:803-809.

90. Singer II, Kawka DW, Schloemann S, Tessner T, Riehl T, Stenson WF. Cyclooxygenase 2 is induced in colonic epithelial cells in inflammatory bowel disease. Gastroenterology 1998;115:297-306.

91. Hadas E, Mazur T. Biosynthesis of prostaglandins in pathogenic and nonpathogenic strains of *Acanthamoeba* spp. Parasitol Res 1997;83:298-299.

92. Kubata BK, Eguchi N, Urade Y, Yamashita K, Mitamura T, Tai K, Hayaishi O, Horii T. *Plasmodium falciparum* produces prostaglandins that are pyrogenic, somnogenic, and immunosuppressive substances in humans. J Exp Med 1998;188:1197-1202.

93. Fusco AC, Salafsky B, Kevin MB. *Schistosoma mansoni:* eicosanoid production by cercariae. Exp Parasitol 1985;59:44-50.

94. Nevhutalu PA, Salafsky B, Haas W, Conway T. *Schistosoma mansoni* and *Trichobilharzia ocelllata*: comparison of secreted cercarial eicosanoids. J Parasitol 1993;79:130-133.

95. Leid RW, McConnell LA. Thromboxane A_2 generation by the larval cestode, *Taenia taeniaeformis*. Clin Immunol Immunopathol 1983;11:317-323

96. Leid RW, McConnell LA. PGE_2 generation and release by the larval stage of the cestode, *Taenia taeniaeformis*. Prostaglandins Leukotrienes Med 1983;11:317-323.

97. Fukushima T, Isobe A, Hojo N, Shiwaku K, Yamane Y, Torii M. The metabolism of arachidonic acid to prostaglandin E_2 in plerocercoids of *Spirometra erinacei*. Parasitol Res 1993;79:634-638.

98. Liu LX, Serhan CN, Weller PF. Intravascular filarial parasites elaborate cyclooxygenase-derived eicosanoids. J Exp Med 1990;172:993-996.

99. Liu LX, Buhlmann JE, Weller PF 1992. Release of prostaglandin E_2 by microfilariae of *Wuchereria bancrofti* and *Brugia malayi*. Am J Trop Med Hyg 1992;46:520-523.

100. Kanesa-thasan N, Douglas JG, Kazura JW. Diethylcarbamazine inhibits endothelial and microfilarial prostanoid metabolism *in vitro*. Mol Biochem Parasitol 1991;49:1-20.

101. Kaiser L, Lamb VL, Tithof PK, Gage DA, Chamberlin BA, Watson JT, Williams JF. *Dirofilaria immitis*: Do filarial cyclooxygenase products depress endothelium-dependent relaxation in the *in vitro* rat aorta. Exp Parasitol 1992;75:159-167.

102. Reiner SL. Parasites and T helper cell development: Some insights. Parasitology Today 1994;10:485-488.

103. Fusco AC, Salafsky B, Delbrook K. *Schistosoma mansoni*: production of cercarial eicosanoids as correlates of penetration and transformation. J Parasitol 1986;72:397-404.

104. Hara I, Hara S, Salafsky B, Shibuya T. *Schistosoma mansoni*: fatty acid-induced cercarial tail loss does not involve eicosanoid production and chemoreceptors. Exp Parasitol 1993;77:484-488.

Section II: Manuscripts I, II, and III

Manuscript I

Entamoeba histolytica Produces Prostaglandin E_2 via a Novel Cyclooxygenase-Like Enzyme: Implications in Pathogenesis and Immunomodulation*

Adam Belley and Kris Chadee

*Submitted to *Molecular Microbiology*

Summary

Entamoeba histolytica is the etiologic agent of amebiasis which is characterized by intestinal ulceration or liver abscess. Little is known of the parasite-derived molecules that facilitate intestinal invasion or enable the parasite to evade host immune responses. In this study, we show that *E. histolytica* produces prostaglandin E_2 (PGE_2), a potent immunomodulator and mediator of inflammation which can play a role in the immunopathogenesis of amebiasis. Amebae produced PGE_2 in a time-dependent manner in the presence of exogenous arachidonic acid, as measured by enzyme immunoassay and corroborated by gas chromatography-mass spectometry. An anti-cyclooxygenase (COX)-1 antiserum cross-reacted with 72 kD and 66 kD amebic nuclear proteins on immunoblots, similar to mammalian COX-1 (68 kD). Both immunoreactive proteins had an isoelectric point (pI) of 4.6, indicating that they may be variants of the same protein and differ from sheep COX-1, which had a pI=6.7. COX activity was enriched in the nuclear fraction of lysed trophozoites and was inhibitable with 1 mM aspirin. These results show that *E. histolytica* produces PGE_2 via a novel COX-like enzyme, which could be used to modulate host responses during infection.

Introduction

The enteric protozoan parasite *Entamoeba histolytica* is a human pathogen that causes widespread morbidity and mortality and ranks behind only malaria and schistosomiasis as leading causes of parasitic-related deaths (1). Infection of the colon results in ulcers, colitis and dysentery. Trophozoites can invade the colonic epithelium and disseminate to the liver or other soft organs to form abscesses. Invasion of the colonic epithelium and evasion of host immune defenses have long been intriguing aspects of amebiasis. Before the organism can bind to the epithelium, it must overcome the protective mucous layer. Amebae bind with high affinity to mucins (2), the high molecular weight glycoproteins that provide the protective gel nature to mucus (3). The parasite elaborates a potent yet unidentified mucin secretagogue (4). It is unknown how *E. histolytica* overcomes the mucous barrier but it was hypothesized that chronic exposure to mucin secretagogues may eventually deplete the stored mucin pool and thus render the mucus less protective (4).

Within tissues, liver granuloma macrophages are deficient in effector and accessory cell functions and consequently are unable to kill amebae (5). *E. histolytica* stimulates host prostaglandin E_2 (PGE_2) production by macrophages (6, 7), a potent immunomodulating agent that down-regulates cytokine production (IL-1, IL-2, IFN-γ and TNF-α) and inhibits Ia molecule expression, key determinants in controlling disease severity and outcome (8-12). PGE_2 is produced by the enzymes cyclooxygenase-1 and -2 (COX-1, -2) from arachidonic acid (AA) which is released from membrane phospholipids by phospholipase A_2. Increased PGE_2 production occurs during most diseases and may exacerbate the inflammatory process and down regulate immune responses. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COX activity and alleviate pain, fever and swelling

associated with increased PGE_2 production (13). PGE_2 also regulates physiologic responses such as causing vasodilation and intestinal Cl^- and mucin secretion (14-16), processes which may contribute to the pathophysiology of amebiasis.

At present, it is unknown whether *E. histolytica* produces immunosuppressive molecules such as PGE_2. Elaboration of this COX- derived product could explain some pathophysiologic symptoms experienced during the intestinal phase of the disease and the immunomodulation that occurs during invasive amebiasis. In this report, we demonstrate that *E. histolytica* produces PGE_2 and characterize a novel COX-like enzyme in the parasite.

Materials and Methods

Cultivation of *E. histolytica*

The virulent strain HM1-IMSS of *E. histolytica* trophozoites was grown axenically in TYI-S-33 medium supplemented with 10% adult bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin sulfate at 36.6°C for 3 d (logarithmic growth phase). Amebae were chilled on ice for 10 min, harvested by centrifugation at 600g for 5 min at 4°C and washed twice in cold Dulbecco's PBS (pH 7.2; Life Technologies, Burlington, Ontario, Canada).

Uptake of [^{3}H]AA

Trophozoites (10^6) were resuspended in 1 ml fresh TYI-S-33 medium containing 0.5 µCi [5,6,8,9,11,12,14,15-^{3}H]AA (150 Ci/mmol; Amersham Pharmacia Biotech, Baie

d'Urfe, Quebec, Canada) and incubated for 1, 2, 4, 6, 12, and 24 h at 36.6°C. Following incubation, amebae were pelleted by centrifugation, washed three times in PBS and resuspended in 5 ml scintillation fluid. [^{3}H]AA uptake was measured by liquid scintillation counting. Nonspecific binding of [^{3}H]AA was determined by incubating glutaraldehyde (4%)-fixed amebae for 6 h under the same conditions.

PGE_2 production by *E. histolytica* trophozoites

Log phase trophozoites were resuspended at a concentration of 10^7/ml in PBS and incubated with 100 μM AA or vehicle for 1, 6 or 12 h at 36.6°C. Following incubation, amebae were pelleted by centrifugation and PGE_2 in the supernatant was extracted with Amprep C2 ethyl columns (Amersham) following the manufacturer's protocol. PGE_2 was quantified with an enzyme immunoassay which uses a monoclonal antibody with very low cross-reactivity (< 0.01%) to other major prostaglandin metabolites (Cayman Chemical Co., Ann Arbor, MI). For GC/MS analysis of parasite-derived PGE_2, trophozoites (10^7) were incubated for 12 h at 36.6°C in 1 ml TYI-S-33 medium containing 2% fetal calf serum (contains undetectable levels of PGE_2). PGE_2 was extracted from supernatant with Amprep columns and processed as described above.

Preparation of amebic nuclear proteins (EhNP)

Amebae were washed twice in PBS and resuspended in lysis buffer (100 mM Tris, pH, 7.4, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml E-64, 10 μg/ml TLCK and 0.5% nonidet P-40 detergent) on ice for 15 min. Nuclei were pelleted by centrifugation at 2000g

for 15 min at 4°C and washed once with lysis buffer. Nuclei were resuspended in sodium phosphate buffer, pH 7.0 and the protein concentration determined by the bicinchoninic acid method (Pierce, Rockford, IL).

Immunoblot analysis of SDS-PAGE resolved proteins

For one-dimensional SDS-PAGE, purified sheep COX-1 (10 ng; Cayman), microsomal proteins from U937 human macrophages (30 μg) and EhNP (30 μg) were boiled in sample buffer containing ß-mercaptoethanol and subjected to SDS-PAGE (10%). For two-dimensional SDS- PAGE, pH gradient (Ampholine; Amersham Pharmacia Biotech) tube gels were prefocused for 1 h at 200 V. The tube gels were loaded with 270 ng purified sheep COX-1 or 200 μg EhNP and run overnight at 700 V. The tube gels were extruded and focused proteins were then separated by SDS-PAGE (10%). The pH gradient was determined in parallel from control tube gels. For immunoblotting, proteins were transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) and blocked in 3% skim milk. Immunodetection consisted of a 1 h incubation with an affinity-purified polyclonal antiserum against sheep COX-1 (241 AP, 1:10,000 dilution, Merck Frosst, Pointe-Claire/Dorval, Quebec, Canada) followed by 1 h with anti-rabbit IgG-horseradish peroxidase-labelled secondary antibody (1:3000; Amersham). Immunoreactive proteins were detected by enhanced chemiluminescence following the manufacturer's protocol (Amersham).

COX activity assays

COX enzyme assays were performed as follows: EhNP were incubated for 1 h at 36.6°C with 100 μM arachidonic acid in sodium phosphate buffer containing 200 μM tryptophan (Sigma Chemical Co., St. Louis, MO) and 2 μM hematin (Sigma) in a total

volume of 500 µl. In reactions involving NSAIDs, 100 µg EhNP were preincubated with either ethanol vehicle, 50 µM indomethacin (INDO; Cayman) or 400 µM or 1 mM aspirin (ASA; Cayman) for 30 min at 36.6°C prior to addition of 100 µM AA (Cayman) and further incubated as described above. The reaction was terminated by the addition of 100 µl of 1 M HCl. PGE_2 was extracted from reaction mixtures with 3 ml of ethyl acetate. The ethyl acetate extracts were dried under a gentle stream of nitrogen and the residues were dissolved in EIA buffer as described by the manufacturer (Cayman). As a control, microsomal proteins (100 µg) were prepared from U937 human macrophages (17) and preincubated for 10 min at 36.6°C with vehicle or 50 µM INDO prior to addition of 20 µM AA for 1 h at 36.6°C.

Gas chromatography/mass spectometry (GC/MS) analysis

High performance liquid chromatography (Pharmacia LKB) using a reverse-phase SuperPac Pep-S (5 µm) 4 x 250 mm column (Amersham Pharmacia Biotech) was used to purify the parasite-derived PGE_2 prior to GC/MS analysis. The flow rate used was 2 ml/min with 27% acetonitrile as a solvent and the absorbance was monitored at 198 nm with a variable wavelength UV monitor (Pharmacia LKB). Samples were resuspended in HPLC solvent and fractions co-eluting with authentic PGE_2 (Cayman) standard (10 µg) were collected and dried prior to GC/MS analysis.

Confirmation of the identity of parasite-derived PGE_2 by GC/MS was done at Merck Frosst Centre for Therapeutic Research (Pointe-Claire/Dorval, Canada). Prostaglandins were derivatized as follows: samples and standards were reacted with 1 ml of 0.1% methoxyamine- hydrochloride (Sigma) dissolved in 0.1 M potassium monophosphate buffer (pH 5-7) at room temperature overnight to form the N-methoxime

of the keto-containing prostaglandins. Products were extracted using Bond Elut C18 solid-phase extraction columns (Varian, Harbor City, CA) following the manufacturer's protocol. Prostaglandins were then eluted with 1 ml methanol in 2 ml silanized glass vials and dried using a Hetovac VR-1 centrifugal evaporation device (High Technology of Scandinavia). Products were reacted with 100 µl of 0.1% diisopropylethylamine (Pierce) and 10% pentafluorobenzylbromide (Pierce) in acetonitrile at 60°C for 15 min to form the pentafluorobenzyl ester derivatives. The products were then dried using the Hetovac. The trimethylsilyl ethers were prepared by resuspending the dried samples in 50 µl of acetonitrile and 50 µl of bis(trimethylsilyl)-trifluoroaceteamide at 60°C for 15 min. The samples were dried and dissolved in a final volume of 25 µl of dodecane. Samples (1 µl) were injected into the gas chromatograph (Hewlett-Packard 5890 Series II) using hot on-column injection at 215°C. A 0.25 mm x 15 m Rt_X-1 capillary column (Restek Corp., Bellefonte, PA) was used with a 0.53 mm x 1 m precolumn (J&W Scientific Inc., Folsom, CA). The oven was heated to 50°C/min to 280°C allowing elution of the PGs in less than 3.5 min. The mass spectrometer (Hewlett-Packard 5988A) was used in resonance electron-capture ionization mode and programmed to monitor the predominant methoxime-pentafluorobenzyl ion of each PG using selected-ion monitoring.

Statistical analysis

Data are presented as mean ± SEM and were analyzed by the Student's *t* test where indicated.

Results

Uptake of [^{3}H]AA and release of PGE_2 by *E. histolytica* trophozoites

Live trophozoites incubated with exogenous [^{3}H]AA, the precursor of PGE_2, showed time-dependent uptake and accumulation of radiolabeled material within the parasites (Table 1). Minimal nonspecific binding of [^{3}H]AA was observed in glutaraldehyde-fixed amebae, indicating that live trophozoites internalized the substrate.

Table 1. Uptake of exogenous [^{3}H]AA by *E.histolytica* trophozoites

Time (h)	[^{3}H]AA cpm
1	13,999 ± 1,980
2	20,142 ± 2,144
4	34,167 ± 3,925
6 (fixed)	3,834 ± 161
6	40,940 ± 9,504
12	53,852 ± 6,219

E.histolytica trophozoites (10^6) were incubated with 0.5 μCi of [^{3}H]AA for the indicated times in 1 ml TYI medium at 36.6°C. Cells were washed 3 times in PBS then resuspended in liquid scintillation fluid and counted. Nonspecific binding of [^{3}H]AA was determined in glutaraldehyde (4%)-fixed amebae incubated for 6 h with the substrate. Data represent the mean ± SEM from 3 independent experiments.

To determine if the parasite converts exogenous AA to PGE_2, the release of PGE_2 by trophozoites incubated with 100 μM AA was quantified over various time points. As shown in Table 2, there was a time-dependent increase in PGE_2 released by amebae over the 12 h time period, as measured by EIA. No measurable PGE_2 was detected after 1 h

from trophozoites incubated without exogenous AA, clearly demonstrating that PGE_2 production was dependent on AA substrate. The identity of the parasite-derived PGE_2 was confirmed by GC/MS analysis.

Table 2. PGE_2 production by *E. histolytica* trophozoites

Time (h)	PGE_2 (pg/ml)
1 (no AA)	0.0
1	28.3 ± 0.9
6	56.7 ± 5.8
12	76.3 ± 6.2

For PGE_2 determinations, trophozoites (10^7) were incubated in 1 ml PBS containing 100 μM AA for the indicated times. No PGE_2 was detected by trophozoites incubated without AA for 1 h (no AA). PGE_2 was extracted with Amprep C2 columns and quantified by EIA. The identity of the parasite-derived PGE_2 was confirmed by GC-MS analysis (data not shown). Data represent the mean ± SEM from 3 independent experiments.

Immunodetection of COX in *E.histolytica*

As PGE_2 production by amebae was highly dependent on the availability of AA substrate, immunoblotting was performed using an affinity-purified polyclonal antibody against sheep COX- 1 to determine if amebae contain an analogous COX-like protein. As shown in Fig. 1, the antibody detected purified sheep COX-1 standard and COX-1 from U937 human macrophages with a molecular mass of 68 kD on one-dimensional SDS-PAGE. An immunodominant protein of 72 kD and a less immunoreactive protein of 66 kD were detected in the nuclear protein fraction (EhNP) from lysed trophozoites (Fig. 1). No reactivity was detected in the cytosolic protein fraction (data not shown). Moreover, no immunoreactivity was observed with pre-immune serum or secondary antibody alone (data

not shown). When EhNP were resolved by two-dimensional SDS-PAGE, the 72 kD and

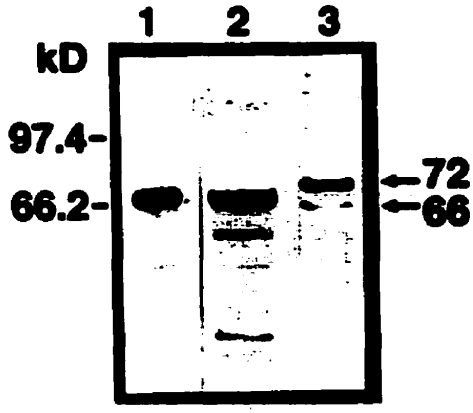

Figure 1. One-dimensional immunoblot analysis of EhNP. Purified sheep COX-1 (10 ng; lane 1), U937 human macrophage microsomes (30 μg; lane 2) and EhNP (30 μg; lane 3), were resolved by SDS-PAGE (10%) and transferred to nitrocellulose. The antibody used was an affinity-purified polyclonal antiserum against sheep COX-1 and immunoreactive proteins were visualized by enhanced chemiluminescence. The antibody detected the sheep and U937 COX-1 at 68 kDa. An immunodominant 72 kD protein and a lesser immunoreactive 66 kD protein were detected in EhNP (arrows).

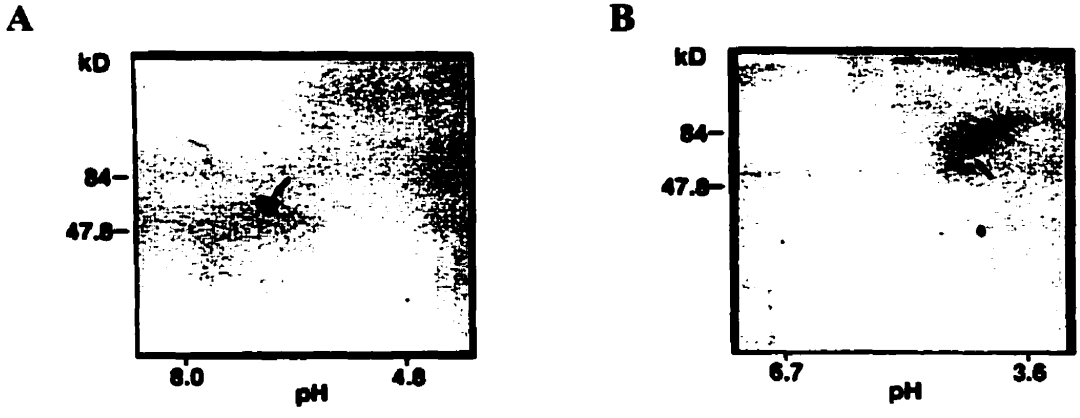

Figure 2. Two-dimensional immunoblot analysis of EhNP. Purified sheep COX-1 (270 ng) and EhNP (200 μg) were initially resolved in pH gradient tubes gels and then by SDS-PAGE (10%). Proteins were then transferred onto nitrocellulose. (A) The affinity-purified polyclonal antiserum against sheep COX-1 detected the 68 kD (arrow) purified sheep COX-1 with a pI of 6.7 whereas in (B), the pI of the immunoreactive 72 and 66 kD proteins (arrows) of EhNP were 4.6. Similar results were obtained from 2 independent experiments.

66 kD proteins had an isoelectric point (pI) of 4.6 (Fig. 2B) whereas purified sheep COX-1 had a pI of 6.7 (Fig. 2A). The identical isoelectric points of both nuclear proteins suggests that they are most likely variant forms of the same protein. Furthermore, the disparity in mass and charge between the COX-like protein in *E. histolytica* and sheep COX-1 indicated that the amebic protein may be a primitive or alternate form of COX.

PGE_2 production by EhNP

To confirm the presence of a COX-like enzyme in EhNP, COX activity was measured in this protein fraction. As shown in Fig. 3, incubation of increasing amounts of EhNP with 100 μM AA substrate caused a dose-dependent increase in the production of PGE_2. No PGE_2 was produced in reactions without EhNP indicating that the transformation of exogenous AA to PGE_2 was by enzymatic conversion. Furthermore, no

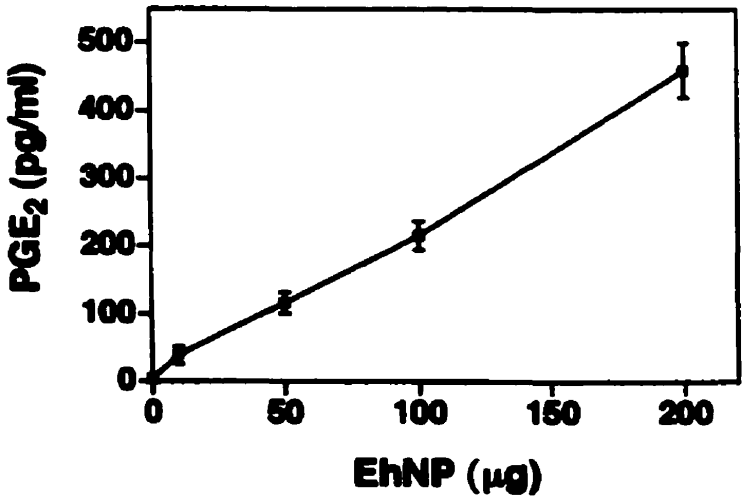

Figure 3. Production of PGE_2 by EhNP. Nuclei from *E.histolytica* were isolated as described in the Materials and Methods. Incubation of increasing amounts of EhNP with 100 μM AA for 1 h at 36.6°C caused a dose-dependent increase in PGE_2 production, as measured by EIA. Data represent the mean ± SEM from 2 independent experiments.

PGE_2 was detected in reactions containing 100 μg of protein from the cytosolic fraction (data not shown). These results demonstrate enriched COX activity within the nuclear protein fraction from lysed trophozoites.

As NSAIDs inhibit mammalian COX activity, PGE_2 production by EhNP was measured following preincubation with INDO or ASA. No significant decreases in PGE_2 production were observed with EhNP preincubated with 50 μM INDO as compared to vehicle treated EhNP ($P > 0.05$; Table 3). A slight but insignificant decrease in PGE_2 levels was measured when EhNP were preincubated with the 400 μM ASA but a significant reduction was observed following incubation with 1 mM ASA (52% inhibition; $P < 0.05$). This indicated that the amebic COX activity differs pharmacologically from mammalian COX-1 in that it is inhibitable with ASA but not with INDO.

Table 3. Inhibition of EhNP COX activity with NSAIDs

NSAID	PGE_2 (pg/ml)
vehicle	231 ± 16
50 μM INDO	203 ± 32
400 μM ASA	198 ± 7.5
1 mM ASA	110 ± 10*

EhNP were prepared as described in Materials and Methods. EhNP (100 μg) were preincubated with NSAIDs or ethanol vehicle for 30 min at 36.6°C prior to addition of 100 μM AA. In control experiments, U937 human macrophage microsomes (100 μg) were prepared as described (17) and preincubated with vehicle or 50 μM INDO for 10 min at 36.6°C prior to addition of 20 μM AA. Production of PGE_2 by vehicle-treated U937 microsomes was 1250 pg/ml whereas pretreatment with 50 μM INDO was 630 pg/ml. PGE_2 production was quantified by EIA. *$P < 0.05$. Data represent the mean ± SEM from 2 independent experiments.

Discussion

We report here the production of the inflammatory mediator PGE_2 by *E. histolytica* and demonstrate that COX activity is enriched in the nuclear fraction of lysed trophozoites. In this regard, the putative amebic COX is similar to the mammalian enzyme, which is localized to the nuclear envelope and the endoplasmic reticulum (18). PGE_2 acts on receptors in the plasma membrane to activate cellular responses in mammalian cells. The nuclear localization of *E. histolytica* COX suggests that parasite-derived PGE_2 may act at the nuclear level to exert its effect. In support of this, PGE_2 receptors (EP) were recently shown to be present in the nuclear envelope of adult rat liver and newborn pig brain cells and activation of the nuclear EP_3 receptor led to increased nuclear Ca^{2+} concentrations and iNOS gene transcription (19). Recently, a prostaglandin transporter was shown to mediate the uptake of prostaglandins and may be a means by which extracellular prostaglandins interact with nuclear receptors (20).

It is apparent that the availability of AA substrate may be a limiting factor for PGE_2 production by the parasite. No PGE_2 was detected after 1 h incubation of trophozoites without exogenous AA. However, longer incubation times may have been required to detect product. Phospholipase A_2 activity was identified in amebae and may provide the means to utilize endogenous AA (21, 22). Moreover, a membrane-associated phospholipase A was also identified and could provide an AA-rich microenvironment during invasive disease (21). Within the host, phospholipase A_2 activity is high during inflammatory states (23) and AA is readily available in inflamed intestinal tissues (24).

We used an affinity purified polyclonal antibody against sheep COX-1 to detect

immunoreactive proteins in amebic protein preparations. Two immunoreactive proteins of 72 kD and 66 kD with the same pI were localized to the nuclear fraction. The similar pI of both amebic proteins indicated that they may be variants of the same protein. The molecular masses of these proteins approximated the mass of mammalian COX-1 (68 kD) but differed in pI (pI = 6.8 for sheep COX-1 as compared to pI = 4.6 for the amebic proteins). Taken together, the nuclear localization of the immunoreactivity, molecular mass and enriched COX activity within the nuclear protein fraction are highly indicative of a putative amebic COX counterpart.

Inhibition of amebic PGE_2 production with ASA but not INDO indicated a pharmacological profile that differed from mammalian COX-1. ASA acetylates the serine residues at positions 529 and 516 of human COX-1 and -2, respectively (25). The serine acetylation site is conserved in all mammalian COX-1 and appears to be conserved in the amebic COX counterpart. In contrast, conversion of AA to prostaglandins by *P. falciparum* was not inhibitable with 3 mM ASA indicating that the serine acetylation site may be absent in this enzyme (26). INDO sterically hinders AA binding in the active site channel of mammalian COX-1 (27). The lack of inhibition of the *E. histolytica* COX activity with a high concentration of INDO (50 μM) leads us to speculate that the differences in the pharmacological profile of the amebic COX may be due to variation of the amino acids near or present at the active site.

The production of PGE_2 by *E. histolytica* may explain some unresolved questions concerning the pathogenesis of intestinal amebiasis and survival of amebae in tissues. In particular, it is unknown how trophozoites overcome the protective mucous layer in the colon. Amebic secretory components caused mucin secretion in rat colon and it was hypothesized that prolonged exposure to secretagogues could eventually deplete the stored mucin pool (4). We recently described the receptor signaling pathway by which PGE_2

stimulates mucin exocytosis in the human colonic epithelial cell line LS174T and rat colon (16). In intestinal amebiasis, neutrophils infiltrate and accumulate in the mucosal layer (28). The recruitment and activation of neutrophils in the lamina propria may cause tissue injury in the pathogenesis of intestinal amebiasis. We recently showed (29, 30) that amebic secretory components and purified PGE_2 stimulated human colonic cells to produce IL-8, a potent chemokine for neutrophils. Thus, we have identified a parasite-derived molecule that may play a direct role in modulating mucin output and IL-8 production, important components involved in modulating epithelial barrier function during intestinal disease.

Amebic liver abscess is the most common extra-intestinal form of infection. During tissue invasion and granuloma formation, macrophages come into contact with trophozoites but are unable to kill these cells. We hypothesize that macrophages in close proximity to the trophozoites encounter PGE_2 released by the parasite, which may down-regulate effector and accessory cell functions. Furthermore, parasite components stimulated naive bone marrow macrophages and hepatic cells to produce elevated levels of PGE_2 (6,7), which may suppress macrophage and lymphocyte functions. In support of this, it was shown that INDO-treatment of hamsters with amebic liver abscess decreased elevated plasma PGE_2 levels to control levels and reduced liver abscess weight by 30% (7).

PGE_2 produced by *E. histolytica* may regulate amebic cellular processes. The localization of the amebic COX activity and the recent discovery of nuclear prostaglandin receptors suggest that PGE_2 signals intracellularly to modulate nuclear functions. Interestingly, PGE_2 and AA induced phagocytosis by the protozoan *Amoeba proteus* (31). The phagocytic response to AA was slightly delayed when compared to PGE_2 and was

inhibitable with INDO, indicating that the substrate must be converted to PGE_2 prior to exerting a response.

The production of PGE_2 by parasitic nematodes, trematodes and protozoans is an overlooked area of research in the pathogenesis of parasitic diseases. Inhibition of parasite COX activity may be a novel and potentially cost effective approach to controlling pathological symptoms associated with parasitic infections. Identifying and characterizing the genes involved in the conversion of AA to PGE_2 has not been done and may be precluded by the disparity of parasite DNA sequences (codon usage and/or low homology). Efforts are currently underway in our laboratory to clone the amebic COX gene.

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References

1. Walsh, J.A. 1986. Problems in recognition and diagnosis of amebiasis: estimation of the global magnitude of morbidity and mortality. *Rev. Infect. Dis.* 8:228-238.

2. Chadee, K., W.A. Petri , D.J. Innes, and J.I. Ravdin. 1987. Rat and human colonic mucins bind to and inhibit adherence lectin of *Entamoeba histolytica*. *J. Clin. Invest.* 80:1245-1254.

3. Forstner, J.F., and G.G. Forstner 1994. Gastrointestinal mucus. *In* Physiology of the gastrointestinal tract. L.R.Johnson, editor. Raven Press, Inc., New York, NY. Volume 2. 3rd edition. 1255-1283.

4. Chadee, K., K. Keller, J. Forstner, D.J. Innes, and J.I. Ravdin. 1991. Mucin and nonmucin secretagogue activity of *Entamoeba histolytica* and cholera toxin in rat colon. *Gastroenterology* 100:986-997.

5. Denis, M., and K. Chadee. 1988. In vitro and in vivo studies of macrophage functions in amebiasis. *Infect. Immun.* 56:3126-3131.

6. Wang, W., and K. Chadee. 1992. *Entamoeba histolytica* alters arachidonic acid metabolism in macrophages in vitro and in vivo. *Immunology* 76:242-250.

7. Sanchez-Ramirez, B., B. Escalante, J.L. Rosales-Encina, and P. Talamas-Rohana. 1997. Role of prostaglandin E_2 in amebic liver abscess formation in hamsters. *Prostaglandins* 53:411-421.

8. Kunkel, S.L., S.W. Chensue, and S.H. Phan. 1986. Prostaglandins as endogenous mediators of interleukin 1 production. *J. Immunol.* 136:186-192.

9. Anastassiou, E.D., F. Paliogianni, J.P. Balow, H. Yamada, and D.T. Boumpas. 1992. Prostaglandin E_2 and other cyclic AMP-elevating agents modulate Il-2 and Il-2Rα gene expression at multiple levels. *J. Immunol.* 148:2845-2852.

10. Kunkel, S.L., M. Spengler, M.A. May, R. Spengler, J. Larrick, and D. Remick. 1988. Prostaglandin E_2 regulates macrophage-derived tumor necrosis factor gene expression. *J. Biol. Chem.* 263:5380-5384.

11. Betz, M., and B.S. Fox. 1991. Prostaglandin E_2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. *J. Immunol.* 146:108-113.

12. Figueiredo, F., R.J. Uhing, K. Okomogi, T.W. Gettys, S.P Johnson, D.O. Adams, and V. Prpic. 1990. Activation of the cAMP cascade inhibits an early event involved in murine macrophage Ia expression. *J. Biol. Chem.* 265:12317-12323.

13. Mitchell, J.A., and T.D. Warner. 1999. Cyclooxygenase-2: pharmacology, physiology, biochemistry and relevance to NSAID therapy. *Br. J. Pharmacol* 128:1121-1132.

14. Willis, A.L. 1987. CRC Handbook of eicosanoids: prostaglandins and related lipids. Volumes 1 and 2 , CRC Press, Inc., Boca Raton, FL. 260 pp.

15. Whittle, B.J.R., and J.R. Vane. 1987. Prostanoids as regulators of gastrointestinal function *In*: Physiology of the gastrointestinal tract. L.R.Johnson, editor. Raven Press, Inc., New York, NY. Volume 1. 2nd edition. 143-180.

16. Belley, A., and K. Chadee. 1999. Prostaglandin E_2 stimulates rat and human mucin colonic mucin exocytosis via the EP_4 receptor. *Gastroenterology* 117:1352-1362.

17. O'Neill, G.P., J.A. Mancini, S. Kargman, J. Yergey, M.Y. Kwan, J.P. Falgueyret, M. Abramovitz, B.P. Kennedy, M. Ouellet, W. Cromlish, S. Culp, J.F. Evans, A.W. Ford-Hutchinson, and P.J. Vickers. 1993. Overexpression of human prostaglandin G/H synthase-1 and -2 by recombinant vaccinia virus: inhibition by nonsteroidal anti-inflammatory drugs and biosynthesis of 15-hydroxyeicosatetraenoic acid. *Mol. Pharmacol.* 45:245-254.

18. Spencer, A.G., J.W. Woods, T. Arakawa, I.I. Singer, and W.L. Smith. 1998. Subcellular localization of prostaglandin endoperoxide H synthases-1 and -2 by immunoelectron microscopy. *J. Biol. Chem.* 273:9886-9893.

19. Bhattacharya, M., K. Peri, A. Ribeiro-da-Silva, G. Almazan, H. Shichi, X. Hou, D.R. Varma, and S. Chemtob. 1999. Localization of functional prostaglandin E_2 receptors EP_3 and EP_4 in the nuclear envelope. *J. Biol. Chem.* 274:15719-15724.

20. Lu, R., N. Kanai, Y. Bao, and V.L. Schuster. 1996. Cloning, in vitro expression, and tissue distribution of a human prostaglandin transporter cDNA (hPGT). *J. Clin. Invest.* 98:1142-1149.

21. Long-Krug, S.A., K.J. Fischer, R.M. Hysmith, and J.I. Ravdin. 1985. Phospholipase A enzymes of *Entamoeba histolytica*: description and subcellular localization. *J. Inf. Dis.* 152:536-540.

22. Vargas-Villarreal, J., A. Olvera-Rodriguez, B.D. Mata-Cardenas, H.G. Martinez-Rogriguez, S. Said-Fernandez, and A. Alagon-Cano. 1998. Isolation of an *Entamoeba*

histolytica intracellular alkaline phospholipase A_2. *Parasitol. Res.* 84:310-314.

23. Balsinde, J., M.A. Balboa, P.A. Insel, and E.A. Dennis. 1999. Regulation and inhibition of phospholipase A_2. *Ann. Rev. Pharmacol. Toxicol.* 39:175-189.

24. Morita, H., K. Nakanishi, T. Dohi, E. Yasugi, and M. Oshima. 1999. Phospholipid turnover in the inflamed intestinal mucosa: arachidonic acid-rich phosphatidyl/plasmenyl-ethanolamine in the mucosa in inflammatory bowel disease. *J. Gastroenterol.* 34:46-53.

25. Lecomte, M., O. Laneuville, C. Ji, D.L. DeWitt, and W.L. Smith. 1994. Acetylation of human prostaglandin endoperoxide synthase-2 (cyclooxygenase-2) by aspirin *J. Biol. Chem.* 269:13207-13215.

26. Kubata, B.K., N. Eguchi, Y. Urade, K. Yamashita, T. Mitamura, K. Tai, O. Hayaishi, and T. Horii. 1998. *Plasmodium falciparum* produces prostaglandins that are pyrogenic, somnogenic, and immunosuppressive substances in humans. *J. Exp. Med.* 188:1197-1202.

27. Callan, O.H., O.-Y. So, and D.C. Swinney. 1996. The kinetic factors that determine the affinity and selecttivity for slow binding inhibition of human prostaglandin H synthase 1 and 2 by indomethacin and flurbiprofen. *J. Biol. Chem.* 271:3548-3554.

28. Prathap, K., and R. Gilman. 1970. The histopathology of acute intestinal amebiasis. A rectal biopsy study. *Am. J. Pathol.* 60:229-246.

29. Yu, Y., and K. Chadee. 1997. *Entamoeba histolytica* stimulates interleukin 8 from human colonic epithelial cells without parasite-enterocyte contact. *Gastroenterology* 112:1536-1547.

30. Yu, Y., and K. Chadee. 1998. Prostaglandin E_2 stimulates interleukin-8 gene expression in human colonic epithelial cells by a posttranscriptional mechanism. *J. Immunol.* 161:3746-3752.

31. Prusch, R.D., S.-M. Goette, and P. Haberman 1989. Prostaglandins may play a signal-coupling role during phagocytosis in *Amoeba proteus*. *Cell Tissue Res.* 255:553-557.

Connecting Statement I

In manuscript I, we demonstrated that *E. histolytica* produces the lipid mediator PGE_2 which it may use to modulate host responses during infection. Thus, we have identified a potential mucin secretagogue and immunomodulating agent that may have diverse effects in the gut. To better understand the mechanism of how *E. histolytica* stimulates mucin secretion, a suitable *in vitro* model to study mucin-parasite interactions is required. Accordingly, we describe the interaction between LS174T cell mucins and *E. histolytica* and show that the LS174T cells secrete mucins that are biochemically similar to native colonic mucins and serve to protect these cells from amebic adherence and killing.

Manuscript II

Interaction of LS174T human colon cancer cell mucins with *Entamoeba histolytica*: an in vitro model for colonic disease*

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Abstract

Background/Aims: Colonic mucins secreted by goblet cells protect the colon by preventing the attachment of enteric pathogens to the epithelium. *Entamoeba histolytica* overcomes this protective barrier and causes ulcerations, allowing the parasite to disseminate to the liver and form abscesses. An in vitro model is used to study the interaction between *E. histolytica* and colonic mucins. ***Methods:*** Secretory mucins from the colonic adenocarcinoma cell line LS174T were collected and their functions assessed by their ability to inhibit amebic adherence to target cells and killing. The cytoprotective effect of mucus against *E. histolytica* cytolysis of LS174T monolayers was studied at 37°C. ***Results:*** Sepharose 4B column chromatography, metabolic labeling with [3H]glucosamine, cesium chloride density gradient centrifugation, and amino acid and carbohydrate compositional analysis revealed that LS174T cell mucins were typical of native colonic mucins. Mucin O-linked oligosaccharides bound to and inhibited the adherence of amebae to Chinese hamster ovary (CHO) cells. *E. histolytica* killing of CHO cell monolayers occurred rapidly, whereas killing of LS174T monolayers with an intact mucus layer was significantly retarded. ***Conclusions:*** Our results show that colonic mucins serve as the first line of host defense against amebic invasion and provide a useful model to study pathogen-mucin interactions.

Introduction

Colonic mucins are high-molecular-weight glycoproteins secreted by goblet cells. Mucins in general contain a protein core with highly branched O-linked oligosaccharides. These molecules are vital in protecting the gastrointestinal tract by providing the gel nature to mucus. The mucus layer lubricates the colon and limits the diffusion of luminal irritants to the delicate colonic epithelium.

The protein moiety of secretory mucins contains a tandem array of serine and threonine/proline repeat units in which the oligosaccharide chains are attached to hydroxyl groups (1). The sugars of the oligosaccharide branches consist mainly of galactose (Gal), fucose, *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc) and sialic acid. Intestinal microorganisms may colonize by binding via lectins to the sugar residues of mucins. The enteric protozoan parasite *Entamoeba histolytica* binds with high affinity to human and rat colonic mucins (dissociation constant of 8.20×10^{-11} mol/L) via the 170-kilodalton heavy subunit of the Gal/GalNAc adherence lectin (2,3). Amebic adherence to colonic epithelial cells is an absolute prerequisite for parasite cytolytic activity and invasion of the colon (4). Thus colonic mucus is the first line of mucosal host defense against *E. histolytica*. Accordingly, invasive disease is only initiated when amebae overcome the mucus layer, adhere to the epithelial lining, and cause ulcerations. *E. histolytica* releases a potent mucus secretagogue with activities similar to that of cholera toxin and it is hypothesized that amebic secretagogue activity may eventually deplete mature mucin stores facilitating amebic attachment to the underlying colonic mucosa (6,7). The eventual secretion of immature (incomplete glycosylated) or subunit mucins that have diminished lectin binding or gel forming capacity, respectively, may increase mucosal vulnerability (8,9).

Many cell lines have been developed and used to study mucin biochemistry, biosynthesis and regulation of secretion. However, not all of the cell lines display goblet cell morphology and eventually differentiate to or are initially low mucin-producing cells. The colon cancer cell line LS174T perhaps best represents colonic goblet cells with a high constitutive secretion of mucins in culture (10-13). It expresses very high levels of *MUC2* mucin messenger RNA (mRNA) (intestinal goblet cell mucin) (14) with lower expression of *MUC3* mucin mRNA (goblet and absorptive cell mucin). The prominent cell lines T84 and HT29 do not express MUC3 mRNA under normal culture conditions but express *MUC2* mRNA (14). The enterocytic-like cell line Caco-2 expresses both *MUC2* and *MUC3* (14) and has been used to study the interaction between adherent *E. histolytica* trophozoites and intestinal epithelial cells (15,16). However, an in vitro model for amebic invasion of colonic cells with an intact intestinal mucus barrier is lacking. In this study, we investigated the interaction between the mucin-producing cell line LS174T and *E. histolytica* to establish an in vitro model suitable to study the role of mucins in host defense in intestinal amebiasis.

Materials and Methods

Cultivation and harvesting of *E. histolytica*

The virulent strain HM1-IMSS of *E. histolytica* is maintained in our laboratory and passaged regularly through gerbil livers to maintain high virulence. It is grown axenically in TYI-S-33 medium at 36.6°C and harvested during logarithmic growth phase (48-72 h) by chilling on ice for 10 min and pelleted by centrifugation (700 X *g* for 5 min at 4°C).

Cell Culture

The human colonic adenocarcinoma cell line LS174T was obtained from the American Type Culture Collection (ATCC) and cultured in MEM (minimal essential medium, Gibco BRL Life Technologies, Burlington, Canada) containing 10% fetal calf serum (Hyclone Laboratories, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin sulfate and HEPES at 37°C in humidified 5% CO_2, in plastic tissue culture flasks (15 X 10 cm). Culture media was replaced with prewarmed media every three days. A high mucin variant was obtained by serially passing LS174T cells through nude mice (nu/nu BALB/c) as described (17). For metabolic labeling of mucins, cells were grown in 24-well plastic culture plates to 80% confluence (2 X 10^5 cells/well). Chinese hamster ovary (CHO) cells were grown in F12 medium (Gibco) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate. Confluent cultures were harvested by 0.25% trypsin digest.

Preparation of LS174T Mucins

LS174T cells were grown to 80% confluence (3 weeks) in plastic tissue culture flasks. Supernatants were collected twice weekly, centrifuged (1,500 X *g* for 5 min) and dialyzed (molecular weight exclusion of 12,000-14,000) extensively against deionized water at 4°C. The crude samples were lyophilized, resuspended in deionized water and the protein concentration was determined by the Bradford method using bovine serum albumin (BSA; Sigma Chemical Company, St. Louis, MO) as a standard (18). The samples were stored at -20°C until needed.

Metabolic Labeling of LS174T Mucins with ^{3}H-glucosamine

LS174T cells grown in 24-well plates were incubated for 24 h with fresh medium containing 2 μCi/ml [^{3}H]glucosamine (specific activity, 40 Ci/mmole; ICN Biomedicals, Inc., Irvine, CA). The cells were washed 3 times with warm medium then resuspended in 1 ml complete MEM. The supernatants were collected after specific time points, centrifuged to remove cell debris (1,500 X *g* for 5 min) and secreted radiolabeled glycoproteins were precipitated by the addition of an equal volume of 10% trichloroacetic acid containing 1% phosphotungstic acid (TCA/PTA; Sigma). Precipitated material was pelleted by centrifugation (2,000 X *g* for 10 min), solubilized with phosphate-buffered saline (PBS, pH 7.2) and neutralized to pH 7.0 with 0.1 M NaOH.

Sepharose 4B Column Chromatography

Because of their high molecular weight, mucins elute with the void volume of Sepharose 4B (S4B) column chromatography (2). To discern mucin from non-mucin glycoproteins, aliquots of ^{3}H-activity (counts per minute) were analyzed by S4B chromatography. The column (1.0 X 30 cm; Bio-Rad Laboratories, Richmond, CA) was equilibrated with 0.01 M Tris-HCl buffer (pH 8.0) containing 0.001% sodium azide (Sigma) and calibrated using the molecular weight standards: blue dextran (BD; >2 X 10^{6}), thyroglobulin (TG; 669,000), BSA (67,000) and chymotrypsinogen A (CTA; 25,000) (Pharmacia Fine Chemicals, Baie D'Urfé, Canada). The sample was applied, eluted at a flow rate of 7 ml/h and 0.5 ml fractions were collected. A 100 μl aliquot from each fraction was dispensed into individual scintillation vials containing 5 ml liquid scintillation fluid (ICN) and the ^{3}H-activity elution profile was determined by liquid scintillation counting.

For the isolation of unlabeled mucins, 100 mg of the concentrated sample were

eluted through a larger S4B column (2.5 X 50 cm) and the eluate was collected in 4 ml fractions. The fractions were monitored for protein (280 nm) and carbohydrates by the phenol-H_2SO_4 method (19). The mucins that eluted in the void volume were dialyzed against deionized water, lyophilized and stored at 4°C until needed.

Purification of S4B Mucins by Cesium Chloride Density Gradient Centrifugation

Lyophilized S4B void volume mucins were resuspended in 5.0 ml PBS (pH 7.4, containing 0.02% {wt/vol} NaN_3 and 1 mM $MgSO_4$) and contaminating nucleic acids were digested with 100 µg/ml DNase I and bovine RNase III (Sigma) for 14 h at room temperature. After centrifugation at 15,000 X *g* for 30 min to remove the resulting insoluble material, the supernatant was dialyzed against PBS for 24 h at 4°C and cesium chloride (CsCl; Sigma) was added to obtain a starting density of 1.41 g/ml (60% wt/vol). The sample (10 ml) was placed in polyallomer centrifuge tubes (14 X 89 mm; Beckman Instruments Inc., Palo Alto, CA) and ultracentrifuged (SW41 rotor) at 250,000 X *g* for 48 h at 4°C. Eight equal volume fractions were withdrawn from the polyallomer tubes and distributed into preweighed test tubes: the density was determined for each fraction. The CsCl gradient fractions were dialyzed extensively against deionized water for 24 h and protein (280 nm), nucleic acid (260 nm) and carbohydrate (phenol-H_2SO_4 method) content were determined.

Amebic Killing of LS174T monolayers

To determine if an intact mucus layer on the surface of the LS174T cells would retard amebic cytotoxicity, LS174T monolayers (80% confluent) in 24 well plates were

incubated with amebae (0.5, 1 and 2 X 10^5) for 2 h at 37°C. Parallel studies were done using CHO cell monolayers as a control for the absence of a mucus barrier. In some experiments, the LS174T cells were treated with the arylglycoside, benzyl-α-Gal*N*ac (2 mM) for 2 d to inhibit O-linked glycosylation (20,21) or tunicamycin (5 μg/ml) and swainsonine (3 μg/ml) for 24 h to inhibit *N*-linked glycosylation (22,23) before interaction with amebae. After incubation with amebae, the plates were chilled on ice (4°C) and rinsed with ice-cold phosphate buffered saline to remove dead cells and amebae and the intact monolayer fixed with 2.5% glutaraldehyde (Sigma). The monolayer was stained with methylene blue (in 0.1 M borate buffer, pH 8.0) and the dye was extracted with 0.1N HCl. The absorbance was measured at 660 nm with a microtitre plate reader. The percentage of monolayer destroyed was calculated using the equation: [(A_{660} control wells- A_{660} experimental wells) ÷ A_{660} control wells] X 100 (24) where control wells were not exposed to amebae (100% cell viability) in homologous experiments.

Amino Acid and Carbohydrate Compositional Analysis

Sepharose 4B void volume and fraction 6 of CsCl density gradient centrifugation purified mucins were analyzed for amino acid and carbohydrate composition. Amino acid analysis was performed as previously described (7).

The glycosyl composition analysis was done at the University of Georgia, Complex Carbohydrate Research Centre. In brief, the mucin samples were analyzed by preparing the trimethylsilyl derivatives of the methyl glycosides followed by gas chromatography and combined gas chromatography/mass spectrometry analysis for neutral and amino sugars. Trimethylsilyl methyl glycosides were prepared by methanolysis in 1 M HCl in methanol, followed by N-acetylation with pyridine and acetic anhydride (for detection of amino

sugars). Gas chromatography analysis of the trimethylsilyl methyl glycosides was done on a Hewlett Packard 5890 GC using a Supelco DB1 fused silica capillary column. Gas chromatography/mass spectrometry analysis was performed using a Hewlett Packard 5890 GC (boise, ID) coupled to a 5970 mass spectrometry detector.

SDS-PAGE and Immunoblotting

SDS-PAGE and immunoblotting were performed as previously described (7) but with slight modification. The primary antibody used for immunodetection was an anti-CsCl purified LS174T mucin (pooled fractions 6 and 7) rabbit immune serum diluted 1:1,000 in dilution buffer.

Amebic Adherence to Target Cells

The ability of LS174T mucins to inhibit *E. histolytica* trophozoite adherence to CHO cells was measured using a standard protocol as described previously (2). Briefly, amebae were washed in M199s (medium 199 (Gibco BRL), 5.7 mM cysteine, 25 mM HEPES, and 0.5% BSA, pH 6.8) and resuspended at a concentration of 10^5 amebae/ml. The suspensions were incubated for 1 h at 4°C with or without mucin preparations as described. Aliquots of amebae (10^4) in the presence of mucin were resuspended in fresh M199s with CHO cells (2 X 10^5), centrifuged (200 X *g* for 5 min) and incubated at 4°C for 2 h. Amebic adherence to CHO cells in the presence or absence of mucins was determined by counting the number of rosettes (a positive rosette is 3 or more adherent CHO cells to an ameba) formed by >100 amebae.

Statistical analysis

Data (mean ± SD) were analyzed using the Student's *t* test. A *P* value of <0.05 was regarded as statistically significant.

Results

Isolation and Functional Characterization of Secreted Mucins from LS174T Cells

The human colonic adenocarcinoma cell line LS174T synthesizes and secretes mucins in culture (10-13). To isolate high-molecular-weight mucins from LS174T cells, supernatants were collected from cell culture medium and their contents analyzed for mucins by S4B chromatography. Due to their immense size ($>1 \times 10^6$), mucins are excluded from the gel bed of a S4B column and consequently elute in the void volume (2,10). Figure 1A shows the S4B chromatography elution profile of secreted proteins from LS174T cells. The cells secreted high- and low-molecular weight glycoproteins in culture constitutively. Nonmucin components represented the majority of the proteins released by the cells as demonstrated by the high protein content in the excluded fractions (fractions 31-45). A minor protein peak eluted with the blue dextran standard (molecular weight $> 2 \times 10^6$) in the void volume and represents the secreted mucin pool (fractions 18-22).

Amebic adherence to target mammalian cells is inhibited by Gal and GalNAc residues of rat and human colonic mucins (2). To assess the functional role of secreted mucins from LS174T cells, we assayed the effect of LS174T-derived mucins on amebic adherence to CHO cells. As shown in Figure 1A, the S4B void volume mucins maximally inhibited the adherence of trophozoites to CHO cells (10 % of control) as compared to low

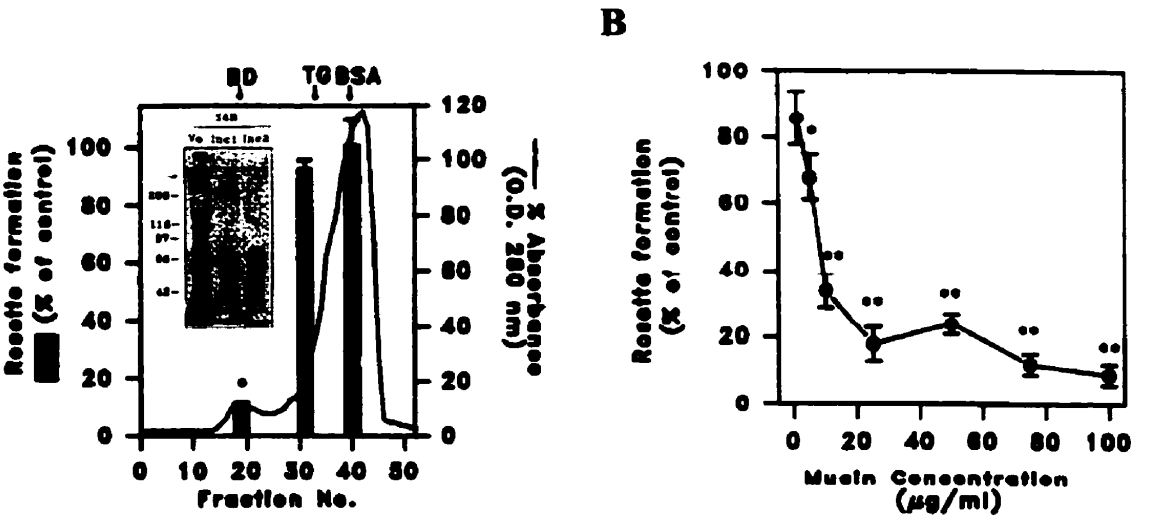


Figure 1. Demonstration of secreted mucins by LS174T cells and inhibition of *E. histolytica* adherence to CHO cells. (A) Crude lyophilized material (100 mg protein) was fractionated on a S4B chromatography column (2.5 x 50 cm) and the eluted fractions were monitored for protein (280 nm). The cells secreted high-molecular-weight mucins that eluted in the void volume. Also shown is the effect of S4B mucins on amebic adherence to CHO cells. Amebae (10^5) were incubated with S4B mucins (100 µg protein/ml) for 1 h at 4°C. Inhibition of adherence of *E. histolytica* trophozoites to CHO cells is shown by the *solid bars*. The S4B void volume mucins significantly (**P* <0.05) inhibited amebic adherence, whereas the included fractions had no effect. (*Inset*) SDS-PAGE (7% gel) of S4B fractions (100 µg protein/lane). High-molecular-weight mucins (silver stain) were present in the S4B void volume (lane V_0) and remained in the stacking gel. No mucins were observed in the included fractions (lanes Incl and Inc2). The arrow indicates the interface between the stacking and separating gels. Molecular weights are shown in the left margin. (B) Concentration-dependent inhibition of amebic adherence to CHO cells by S4B void volume purified LS174T mucins (µg protein/ml). Rosette formation decreased significantly (**P* <0.05, ***P* <0.01) with increasing mucin concentrations.

molecular weight proteins (Incl and Inc2, 95-100 % of control) which had no effect on amebic adherence (100 µg protein for each). S4B void volume mucins significantly (*P* <0.05 to *P* <0.01) inhibited amebic adherence in a dose dependent manner; only 10%-20% of amebae were positive for rosette formation at a mucin concentration between 5-100 µg protein/ml (Figure 1B). As shown in Figure 1A (inset), the void volume mucins were relatively free of contaminating low-molecular-weight proteins and contained mostly high-molecular-weight mucins that were retained in the stacking gel after 7% SDS-PAGE. In contrast, the included fractions contained a variety of low-molecular-weight proteins and

were devoid of mucins. [^{3}H]glucosamine metabolic labeling studies revealed that >90% of the LS174T ^{3}H-labeled glycoproteins eluted in the void voulme of a S4B column and when subjected to 7% SDS-PAGE followed by fluorography, the labeled void volume mucins were retained in the stacking gel (data not shown). These results indicate that LS174T cells secrete functionally active mucins and that colonic mucins may serve to protect against the adherence of intestinal microorganisms to the epithelium.

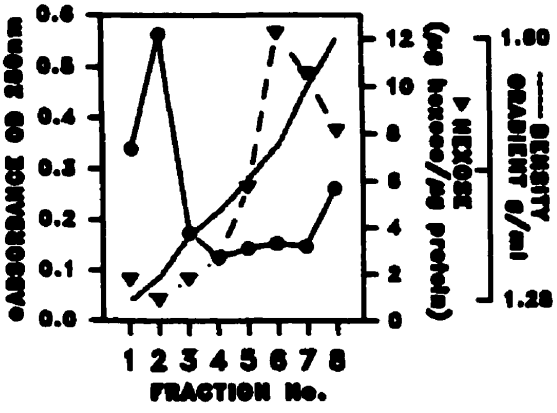
Purification of LS174T Mucins by CsCl Density Gradient Centrifugation

Mucins purified by CsCl density gradient centrifugation separates the noncovalently bound mucins from other proteins in the high-density fractions. As shown in Figure 2A, the S4B void volume nuclease digested mucins subjected to CsCl purification resulted in eight glycoprotein fractions with different densities and protein and carbohydrate content. The colonic mucins were found exclusively in the high density (>1.46 g/ml) hexose rich fractions (fractions 6-8). Similarly, when subjected to CsCl density gradient centrifugation, ^{3}H-metabolically labeled S4B void volume mucins showed peak ^{3}H-activity (Figure 2B) in fractions 6 and 7 (densities 1.46 and 1.52 g/ml, respectively). The CsCl fractions were analyzed by SDS-PAGE and visualized by silver staining (Figure 2C). Staining was diffuse but intense in the stacking gel wells containing the high density CsCl fractions (fractions 6-8). Accordingly, fluorography of SDS-PAGE resolved ^{3}H-glucosamine-labeled glycoproteins showed activity only within the stacking gel wells of these CsCl fractions (Figure 2B, inset). A stained protein of similar molecular weight to the putative link peptide (26) (mol wt of 118,000) was observed in fractions 6 and 7 (Figure 2C). Many low-molecular-weight proteins were observed in all fractions. However, antimucin antiserum raised against fraction 6 and 7 detected mucins exclusively

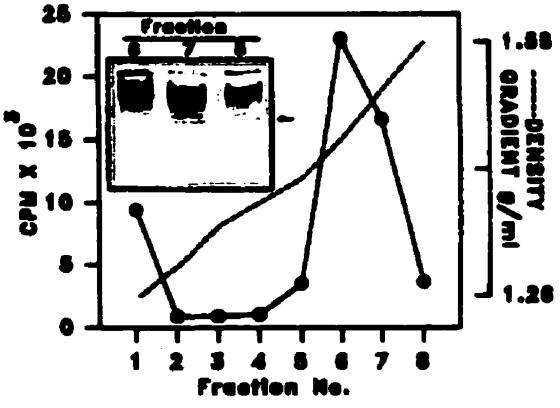
in the stacking gel lanes containing the high density CsCl fractions (fractions 6-8; Figure 2D). No cross reactivity was observed with low-molecular-weight proteins, showing that the antibody recognizes only components associated with high-molecular-weight mucins. These results indicate that mucins are primarily partitioned in fractions 6-8 of the CsCl gradient.

The CsCl fractions (1 μg protein/ml) were assayed for their ability to inhibit amebic adherence to CHO cells (Table 1). Fraction 6 inhibited (P <0.05) rosette formation the most effectively (14 ± 5.0 % of control). Rosette formation was affected to a lesser extent by fractions 7 and 8 (42 ± 9.3 % and 55 ± 6.4 %, respectively), whereas the lower-density fractions (fractions 1-5) had no significant effect on adherence. When fraction 6 was assayed for its inhibitory effect on adherence, inhibition occurred in a dose dependent manner; rosette formation was significantly inhibited (P <0.05) at a concentration as low as 100 ng protein/ml (78%; Figure 3).

A



B



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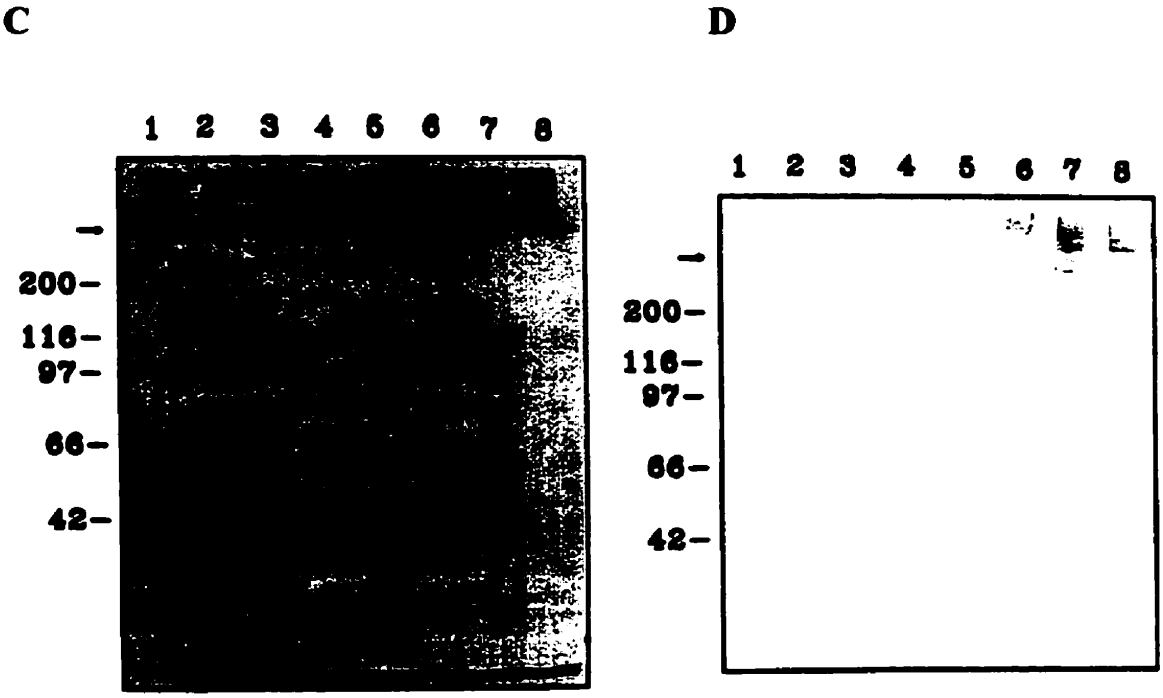

Figure 2. CsCl density gradient centrifugation of LS174T-derived mucins. Lyophilized labelled and unlabeled S4B void volume (6 mg protein) mucins were resuspended in phosphate-buffered saline and treated with nucleases; then CsCl was added to a starting density of 1.41 g/ml. After ultracentrifugation at 4°C (250,000 X *g* for 48 h), eight equal volume fractions were withdrawn and weighed. Carbohydrate, nucleic acid (absorbance at 260 nm) and protein content (absorbance at 280 nm) were determined for each fraction. (A) S4B void volume mucins were localized to the high density fractions ($1.46 > d < 1.60$ g/ml; fractions 6-8) as demonstrated by the high hexose (phenol-H_2SO_4 assay) and low protein (measured as absorbance at 280 nm) in these fractions. (B) Partitioning of the ^{3}H-labeled S4B void volume mucins (5.5 X 10^6 cpm) to the high density fractions (fractions 6-8) of the CsCl gradient. ^{3}H-activity of labeled mucins peaked in fraction 6 with decreasing activity in fractions 7 and 8. (*Inset*) Fluorograph of the high density CsCl fractions (1 X 10^4 cpm/lane) resolved by SDS-PAGE (7% gel). The radiolabeled mucins were retained in the stacking gel. The *arrow* indicates the interface between the stacking and separating gels. (C, D) Determination of mucins by SDS-PAGE and immunoblotting. The CsCl fractions (1 μg protein/lane) were resolved by SDS-PAGE (7% gel), stained with silver or transferred to nitrocellulose membranes and probed with an anti-mucin antibody as described. Silver staining revealed that mucins were primarily concentrated in fraction 6 but were also apparent in fractions 7 and 8. The anti-mucin immune serum raised against CsCl purified mucin fractions 6 and 7 reacted with mucins in fractions 6, 7 and 8 in the stacking gel. No reactivity was observed in fraction 1-5 or with low-molecular-weight proteins in the running gel. The *arrow* indicates the interface between the stacking and separating gels. Molecular weights are shown in the left margin.

Table 1. CsCl purified LS174T mucins inhibit *E. histolytica* adherence to CHO cells

CsCl fractions (1 μg/ml)	n	Rosette formation (% of control) ± SD
1	6	97 ± 6.4
2	6	89 ± 9.6
3	6	100 ± 6.5
4	6	94 ± 1.2
5	6	88 ± 6.4
6	6	14 ± 5.0*
7	6	42 ± 9.3*
8	6	55 ± 6.4*

*P <0.01 by Student's t test compared with homologous controls in the absence of mucin

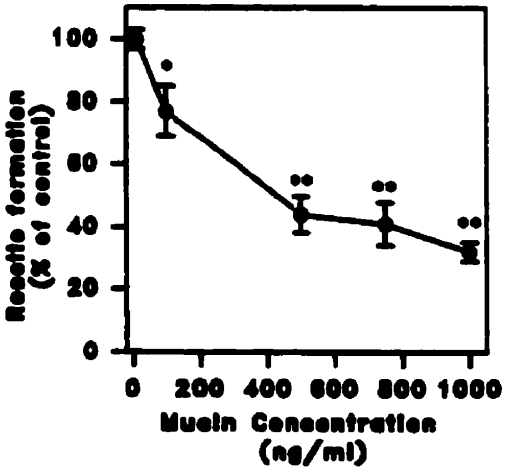

Figure 3. Effect of CsCl density gradient centrifugation purified S4B void volume mucins on *E. histolytica* adherence to CHO cells. Approximately 10-fold less CsCl-purified mucins than S4B void volume mucins were needed to equivalently inhibit amebic adherence to CHO cells. (*P <0.05, **P <0.01 compared with homologous controls).

It was shown by enzymatic digestion with various glycosidases that digest proteoglycans that the high-molecular-weight proteins (labeled with [^{3}H]glucosamine) secreted by LS174T cells, are mucins an not proteoglycans (13). To verify that in fact mucins and not contaminating proteoglycans are the inhibitory components, fraction 6 was labeled with ^{125}I (2,3) and subjected to enzymatic digestion. Hyaluronidase and chondroitinase ABC had no effect on the elution profiles of the ^{125}I-mucins that eluted in the void volume of a S4B column. However, pronase completely degraded the ^{125}I-void volume mucins, which subsequently eluted in the included fractions of S4B (data not shown). These data confirm that fraction 6 contains mucins that inhibit amebic adherence to CHO cells and that it contains no contaminating proteoglycan.

Amino Acid and Carbohydrate Compositional Analysis of LS174T Mucins

Intestinal mucins are characterized by a high serine, threonine, and proline content and O-linked oligosaccharides (1). Mucins from S4B void volume and CsCl fraction 6 were analyzed for amino acid composition. On a molar percent basis, the serine and threonine content (9.65 ± 0.16 and 20.73 ± 0.38, respectively) of fraction 6 was high, totalling 30%. The proline content was also high at 14.04 ± 0.63%. S4B void volume mucins contained lower than expected values for serine, threonine and proline (8.21 ± 0.21, 9.83 ± 0.47 and 8.60 ± 0.25, respectively) suggesting perhaps the presence of contaminating nonmucin glycoproteins. The carbohydrate compositional analysis of S4B void volume and CsCl fraction 6 was typical for mucins with high GalNac, GlcNAc and Gal content (Table 2). Of note is the enrichment of GlcNAc and decreased glucose content of the CsCl-purified mucins, suggesting that a non-mucin glycoprotein may be present in the S4B void volume fraction. Overall, the expected amino acid and carbohydrate contents

for fraction 6 were in accordance with a previously published report (10) and corroborate the presence of mucins in the S4B void volume and fraction 6.

Table 2. Carbohydrate composition of secreted mucins from LS174T cells

	Molar percent	
Glycosyl residue	S4B void volume	CsCl fraction 6
GalNAc	18	15
GlcNAc	24	46
Galactose	20	21
Glucose	10	3
Fucose	13	9
Sialic acid	5	2
Mannose	5	3
Xylose	3	2
Rhamnose	2	Nil

Interaction between LS174T Monolayers and *E. histolytica*

Having established that soluble mucins produced by LS174T cells can inhibit *E. histolytica* adherence to heterologous cells, we determined if an intact mucus layer produced by confluent LS174T cells can prevent epithelial cell destruction by amebae. To address this issue, the destruction of nonmucin-producing CHO cell monolayers was compared to mucin-secreting LS174T cell monolayers. As shown in Figure 4, CHO cell monolayers were significantly (P <0.01) disrupted over a 2 h incubation period with increasing concentration of *E. histolytica* trophozoites. In contrast, LS174T cell monolayers were only slightly affected at the highest inoculum used; the lower inocula had no effect. To show that mucins were the major protective component secreted by LS174T cells in this interaction, we treated the cells with the O-linked glycosylation inhibitor

benzyl-α-GalNAc or the N-linked glycosylation inhibitors tunicamycin or swainsonine. The inhibition of O-linked glycosylation resulted in almost complete destruction (P<0.01) of the LS174T monolayers whereas tunicamycin slightly rendered the cells susceptible to lysis (P <0.05) by amebic trophozoites (Figure 5). Swainsonine had no effect on monolayer vulnerability. These results clearly show that an intact mucus layer and its mucin component is a potent epithelial barrier protecting the cellular integrity of LS174T monolayers.

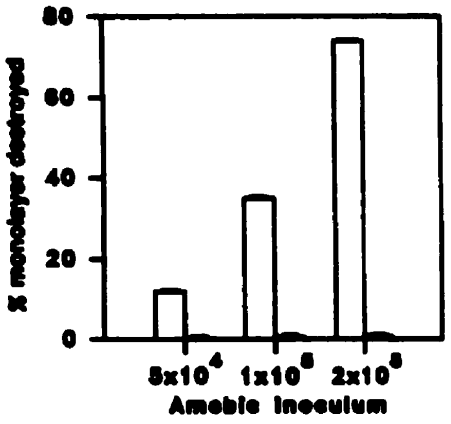

Figure 4. Killing of mammalian cell monolayers by *E. histolytica*. CHO ( ) and LS174T cell monolayers (■) were incubated with various inocula of amebae (0.5, 1 and 2 X 10^5) for 2 h at 37°C. Destruction was determined by the colorimetric assay. CHO cell monolayers were highly susceptible to lysis by *E. histolytica* (12%, 35% and 74% destroyed, P <0.05) over the time period whereas only the highest inoculum of amebae used slightly affected LS174T monolayers (0.6% destroyed, P <0.05).

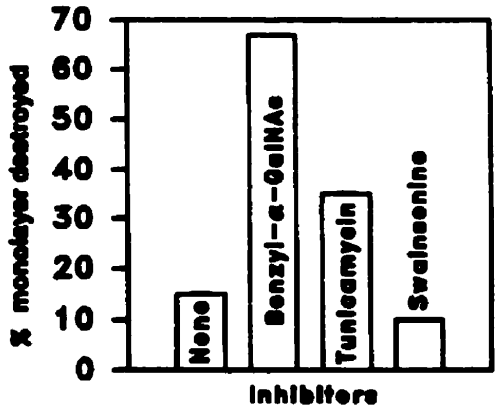

Figure 5. The effect of O- and N-linked glycosylation inhibitors on susceptibility of LS174T monolayers to lysis by *E. histolytica*. LS174T cells were grown to confluence and then treated with glycosylation inhibitors for 24 h at 37°C. The monolayers were then incubated with 10^5 amebae for 2 h at 37°C. Sixty-seven percent of the monolayer was destroyed by the amebae after treatment of LS174T cells with the O-linked glycosylation inhibitor benzyl-α-GalNAc (P <0.05). The N-linked glycosylation inhibitors tunicamycin (P <0.05) and swainsonine caused 35% and 10% of the monolayer to be lysed by *E. histolytica*, respectively.

Discussion

The constitutive production of mucus serves many purposes and is presumed be the first line of defense against colonic invasion by pathogens. Enteric pathogens that bind colonic mucins are sloughed into the lumen and swept out of the intestine in stool. Mucins may provide the necessary anchor for colonization but simultaneously deny access to the colonic epithelium. In amebic infections, invasion and cytolysis of colonic cells by *E. histolytica* absolutely requires contact. Amebic adherence to target cells is mediated by the 170 kDa heavy subunit of the Gal/Gal*N*Ac adherence lectin (2,3). Human and rat colonic mucins bind with high affinity to the lectin and inhibit its function (2). Mucins from these sources are difficult to obtain for use in in vitro studies. Moreover, animal models used to elucidate the function of mucins in enteric infections are laborious. It was the aim of this study to develop an adequate in vitro model to study the role of mucins in enteric infections.

The production of mucins by the colonic adenocarcinoma cell line LS174T is well documented (10-13). However, it is not known if the secreted mucins and the adherent mucus layer on the surface of the cells are functional. Our data clearly show that LS174T cells secrete high-molecular-weight mucins based on the following. (1) The S4B chromatography elution profile of LS174T secretory products yielded high-molecular-weight protein that eluted with the void volume and the majority of ^{3}H-activity from metabolically labeled glycoproteins also eluted in the void volume. (2) Unlabeled and labeled S4B void volume material purified by CsCl density gradient centrifugation yielded high-molecular-weight glycoprotein in the high-density fractions when analyzed by SDS-PAGE. (3) Digestion with pronase caused the CsCl purified mucins to elute with the included fractions whereas treatment with enzymes that digest proteoglycans had no effect

on the elution profile of the S4B void volume material. (4) Carbohydrate and amino acid analysis of S4B void volume and CsCl fraction 6 yielded expected values for serine, threonine and proline with typically high content of Gal, GalNAc and GlcNAc.

Our next objective was to demonstrate a functional relevance of LS174T-derived mucins. It was previously shown that amebic adherence to target cells is inhibited by Gal monomers and purified human and rat colonic mucins (2). Because biochemical analysis showed that LS174T-derived mucins are similar to human colonic mucins, we assayed for their ability to inhibit amebic adherence to target cells. Our data demonstrate that S4B void volume mucins obtained from LS174T cells almost completely inhibited amebic adherence. Furthermore, purification of S4B void volume mucins by CsCl density gradient centrifugation decreased the concentration of mucins needed to equivalently inhibit amebic adherence by approximately 10-fold.

The killing of mammalian cells by *E. histolytica* occurs indiscriminately, only after contact is made. To address whether a mucus barrier is protective, we assayed the killing of nonmucin (CHO) and mucin producing (LS174T) cells by *E. histolytica*. Destruction of CHO cell monolayers occurred at the lowest concentration of amebae used (5×10^4) and monolayers were almost completely disrupted (74% ± 0.12) by 2×10^5 amebae. In contrast, mucin-producing LS174T cell monolayers were unaffected by the lower inoculum of amebae and only minutely affected at the highest concentration of amebae. We believe that the intact mucus layer and the mucins secreted by the LS174T cells are responsible for protecting the monolayer integrity by preventing the binding of amebae to the monolayers. To show that indeed mucins are protective, we pretreated the LS174T cells with glycosylation inhibitors to eliminate the binding capacity of the mucins and impede its barrier function. The N-linked glycosylation inhibitor tunicamycin modestly increased LS174T cell monolayer vulnerability to lysis by *E. histolytica*. Swainsonine, which also

inhibits N-linked glycosylation, did not affect LS174T cell monolayer vulnerability. It has been suggested that N-linked glycans of mucin protein cores may be involved in the polymerization of subunit mucins into polymeric high molecular weight mucins (1) and thus subunit mucins may not be as protective. The varying destruction of monolayers after pretreatment of cells with the two N-linked glycosylation inhibitors may reflect the mechanisms by which tunicamycin and swainsonine inhibit glycosylation (27). The O-linked glycosylation inhibitor benzyl-α-GalNac had a pronounced effect on LS174T cell monolayer destruction. The lack of O-linked sugars on mucins secreted by pretreated cells rendered the monolayers susceptible to lysis by *E. histolytica*; thus, the functional moiety of mucins that inhibit amebic adherence are the O-linked glycans.

The ability of *E. histolytica* to overcome the mucus blanket is a poorly understood phenomenon in amebiasis research. Whether the parasite elaborates a mucinase activity or depletes the mucin stores through secretagogue activity has been contemplated (6,7). We have previously shown that *E. histolytica* releases potent mucus secretagogues (7) and that mucin depletion occurs prior to amebic invasion of the cecal wall in the gerbil model for intestinal amebiasis (4). We believe the model described here can be used to address these issues in vitro and likewise to study the interaction between other enteric pathogens and colonic mucins.

In summary, mucins secreted by the colonic adenocarcinoma cell line LS174T were effective at inhibiting amebic adherence to mammalian tissue culture cells. An intact mucus layer was shown to be effective at protecting LS174T cell monolayers from lysis by amebic trophozoites. Our results support the role of mucins as being the first line of host defense against colonic invasion and provides a useful model to study the interaction between enteric pathogens and mucins in epithelial barrier functions.

References

1. Forstner JF, Forstner GG. In: Johnson LR, ed. Physiology of the gastrointestinal tract. Volume 2. 3rd ed. New York: Raven. 1994:1255-1283.

2. Chadee K, Petri WA, Innes DJ, Ravdin JI. Rat and human colonic mucins bind to and inhibit adherence lectin of *Entamoeba histolytica*. J Clin Invest 1987;80:1245-1254.

3. Chadee K, Johnson ML, Orozco E, Petri WA, Ravdin JI. Binding and internalization of rat colonic mucins by the galactose/N-acetyl-D-galactosamine adherence lectin of *Entamoeba histolytica*. J Infect Dis 1988;398-406.

4. Chadee K, Meerovitch E. *Entamoeba histolytica*: early progressive pathology in the cecum of the gerbil (*Meriones unguiculatus*). Am J Trop Med Hyg 1985;34:283-291.

5. Tse SK, Chadee K. The interaction between intestinal mucus glycoproteins and enteric infections. Parasitol Today 1991;7:163-172.

6. Chadee K, Keller K, Forstner J, Innes DJ, Ravdin JI. Mucin and nonmucin secretagogue activity of *Entamoeba histolytica* and cholera toxin in rat colon. Gastroenterology 1991;100:986-997.

7. Tse SK, Chadee K. Biochemical characterization of rat colonic mucus secreted in response to *Entamoeba histolytica*. Infect Immun 1992;60:1603-1612.

8. Lee SP, Nicholls JF, Robertson AM. Effects of trimoprostil, a prostaglandin E_2 analogue, on human gastric acid secretion and soluble mucin output. Eur J Clin Invest 1987;17:1-6.

9. Phillips TE, Stanley CM, Wilson J. The effect of 16,16-dimethyl prostaglandin E_2 on proliferation of an intestinal goblet cell line and its synthesis and secretion of mucin glycoprotein. Prostaglandins Leukotrienes and Essential Fatty Acids 1993;48:424-428.

10. Byrd JC, Nardelli J, Siddiqui B, Kim YS. Isolation and characterization of colon cancer mucin from xenografts of LS174T cells. Cancer Research 1988;48:6678-6685.

11. Kuan SF, Byrd JC, Basbaum CB, Kim YS. Characterization of quantitative mucin variants from a human colonic cancer cell. Cancer Research 1987;47:5714-5724.

12. Boland CR, Roberts JA, Siddiqui B, Byrd J, Kim YS. Cancer-associated colonic mucin in cultured human tumor cells and athymic (nude) mouse xenografts. Cancer Res1986;46:5724-5729.

13. Siddiqui B, Byrd JC, Fearney FJ, Kim YS. Comparison of metabolically labeled mucins of LS174T human colon cancer cells in tissue culture and xenografts. Tumor Biol 1989;10:83-94.

14. Hollingsworth MA, Strawhecker JM, Caffrey, TC, Mack DR. Expression of MUC1, MUC2, MUC3 and MUC4 mucin mRNAs in human pancreatic and intestinal tumor cell lines. Int J Cancer 1994;57:198-203.

15. Rigothier MC, Coconnier MH, Servin AL, Gayral P. A new in vitro model of *Entamoeba histolytica* adhesion, using the human colon carcinoma cell line Caco-2: scanning electron microscopic study. Infect Immun 1991;59:4142-4146.

16. Li E, Stenson WF, Kunz-Jenkins C, Swanson PE, Duncan R, Stanley SL. *Entamoeba histolytica* interactions with polarized human intestinal Caco-2 epithelial cells. Infect Immun 1994;62:5112-5119.

17. Keller K, Olivier M, Chadee K. The fast release of mucin secretion from human colonic cells induced by *Entamoeba histolytica* is dependent on contact and protein kinase C activation. Arch Med Res 1992;23:217-221.

18. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-254.

19. Dubois M, Giles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28:350-356.

20. Kuan SF, Byrd JC, Basbaum C, Kim YS. Inhibition of mucin glycosylation by aryl-*N*-acetyl-α-galactosaminides in human colon cancer cells. J Biol Chem 1989;264:19271-19277.

21. Bresalier RS, Niv Y, Byrd JC, Duh, Qy, Toribara NW, Rockwell RW, Dahiya R, Kim YS. Mucin production by human colonic carcinoma cells correlates with their metastatic potential in animal models of colon cancer metastasis. J Clin Invest

1991;87:1037-1045.

22. Bharathan S, Moriarty J, Moody CE, Sherblon AP. Effect of tunicamycin on sialomucin and natural killer susceptibility of rat mammary tumor ascites cells. Cancer Res 1990;50:5250-5256.

23. Newton SA, White SL, Humphries MJ, Olden K. Swainsonine inhibition of spontaneous metastasis. J Natl Cancer Inst 1989;81:1024-1028.

24. Göttke M, Chadee K. Exogenous nitric oxide stimulates mucin secretion from LS174T colonic adenocarcinoma cells. Inflamm Res 1996;45:209-212.

25. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979;76:4350-4354.

26. Fahim REF, Specian RD, Forstner GG, Forstner JF. Characterization and localization of the putative `link' component in rat small-intestinal mucus. Biochem J 1987;243:631-640.

27. Elbein AD. Glycosylation inhibitors for N-linked glycoproteins. Methods Enzymol 1987;138:661-709.

Connecting Statement II

In manuscript I, we demonstrated that *E. histolytica* produces PGE_2, a potential mucin secretagogue released by the parasite. In manuscript II, we described the interaction between *E. histolytica* and LS174T cell mucins to provide a suitable *in vitro* model for intestinal disease and to study the mechanism whereby mucin secretion is regulated. Intestinal inflammation is characterized by increased production of PGE_2 which in turn, may serve to regulate several physiologic and immune functions. In manuscript III, we describe the receptor-coupled mechanism by which PGE_2 modulate mucin secretion to gain a better understanding of how mucin secretion is regulated during inflammation and in *E. histolytica* infections.

Manuscript III

Prostaglandin E_2 Stimulates Rat and Human Colonic Mucin Exocytosis via the EP_4 Receptor*

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Abstract

Background & Aims: Mucins form an integral part of innate host defenses against intestinal pathogens and irritants. However, the mechanisms whereby mucin secretion is regulated during inflammation are poorly understood. Because prostaglandin E_2 (PGE_2) is prominent during intestinal inflammation, we investigated its receptor-signaling pathway coupled to mucin exocytosis in the colonic epithelial cell line LS174T and rat colon. ***Methods:*** Reverse transcription-polymerase chain reaction and [^{3}H]PGE_2 binding assays were used to identify the PGE_2 receptors (EP). Intracellular cyclic adenosine monophosphate ($[cAMP]_i$) was quantified by enzyme immunoassay. Mucins were metabolically labeled with [^{3}H]glucosamine, and mucin secretion quantified by Sepharose 4B column chromatography, immunoblot analysis, and cesium chloride density gradient centrifugation. ***Results:*** RT-PCR and DNA sequence analysis identified EP_2, EP_3, and EP_4 receptors. Mucin secretion and $[cAMP]_i$ production by LS174T cells were stimulated dose-dependently by PGE_2, the EP_4 receptor agonist 1-OH-PGE_1 and the EP_3/EP_4 agonist M&B28767 and were inhibited with the adenylate cyclase inhibitor SQ22536. The EP_1, EP_2 and EP_3/EP_1 receptor agonists iloprost, butaprost and sulprostone, respectively, had no effect. Similar results were obtained in rat colonic loop studies confirming that the EP_4 receptor is linked to mucin exocytosis in vivo. [^{3}H]PGE_2 binding to cell membranes identified a high affinity binding site which was competitively inhibited by M&B28767 (EP_3/EP_4) > 1-OH-PGE_1 (EP_4) > sulprostone (EP_3/EP_1) > butaprost (EP_2). ***Conclusions:*** PGE_2 coupling to the EP_4 receptor stimulates $[cAMP]_i$-dependent mucin exocytosis.

Introduction

Prostaglandin E_2 (PGE_2) is produced from arachidonic acid by two isoforms of the cyclooxygenase (COX-1, COX-2) enzyme and can modulate a variety of physiological, inflammatory and immunologic functions in mammals.[1,2] Its diverse biological activity can be attributed to four specific G protein-coupled receptors, termed EP, with which it interacts: the EP_1 receptor increases $[Ca^{2+}]_i$ via $G_{\alpha o}$, EP_2 and EP_4 increase $[cAMP]_i$ via $G_{\alpha s}$ and EP_3 decreases $[cAMP]_i$ via $G_{\alpha i}$.[3-8]

Recent studies have shown that EP-receptor messenger RNAs (mRNAs) are distributed throughout the mouse and rat gastrointestinal tracts.[9,10] Several studies have begun to describe the functional role of these receptors in gastrointestinal tissues. Signaling via the EP_1 and EP_3 receptors was shown to stimulate gastric and duodenal bicarbonate secretion.[11] Moreover, a house-keeping Cl- channel in rabbit gastric parietal cells was activated by the EP_3 receptor.[12] Although past studies have shown that PGE_2 was cytoprotective to the gastrointestinal mucosa, its mechanism of action was unknown.[13,14] A new report shows that PGE_2 and prostacyclin act synergistically to restore epithelial barrier function through closure of tight junctions between porcine epithelial cells.[15] Together these studies suggest that the cytoprotective effect of PGE_2 in gastrointestinal tissues involves signaling via several EP receptors.

Intestinal secretory mucins constitute a vital component of the intestinal epithelial barrier. These very large secretory glycoproteins ($M_r > 10^6$) polymerize into viscous

lubricating gels that protect the intestinal epithelium from physical damage or injury caused by chemical or microbial irritants. Although secretory mucins form part of the extrinsic barrier, their release from goblet cells is under tight control. Goblet cells secrete mucins constitutively but also respond to inflammatory and neurologic stimuli (secretagogues) by releasing large amounts of stored mucins.[16] In the present study, we rationalized that EP receptors may contribute to the cytoprotective nature of PGE_2 by coupling to mucin exocytosis. We describe for the first time the receptor and signaling pathway by which PGE_2 evokes mucin secretion.

Materials & Methods

Reagents

PGE_2, PGF_{2a}, 13,14-dihydro-15-keto-PGE_2, sulprostone and $[cAMP]_i$ enzyme immunoassays were obtained from Cayman Chemical Co. (Ann Arbor, MI). 3-Isobutyl-1-methylxanthine (IBMX) was obtained from Calbiochem (La Jolla, CA). 9-Tetrahydro-2-furyl-adenosine (SQ22536) was obtained from Biomol (Plymouth Meeting, PA). Forskolin and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Co. (St. Louis, MI). Iloprost (Dr. F. McDonald, Schering AG, Berlin, Germany), butaprost (Dr. M.P. Kotick, Bayer Corp., West Haven, CT) and M&B28767 (J. Hough, Rhône-Poulenc Rorer Ltd., Dagenham, Essex) were generous gifts from the mentioned suppliers. The plasmids containing the cloned human EP_1, EP_3, and EP_4 cDNAs were obtained from Dr. M. Abramovitz (Merck Frosst, Pointe Claire, Canada). The EP_2 cDNA plasmid was obtained from Dr. K. Kedzie (Allergan, Irvine, CA). Oligonucleotide primers were ordered from Life Technologies (Burlington, Ontario, Canada).

Cell Culture

The human colonic adenocarcinoma cell line LS174T was obtained from the American Type Culture Collection (Rockville, MD) and was cultured in minimal essential medium containing 10% fetal bovine serum (HyClone, Logan, UT), 100 U/ml penicillin, 100 μg/mL streptomycin sulfate and 20 mM Hepes at 37°C in humidified 5% CO_2 in plastic 6 or 24 well tissue culture plates. Culture medium was replaced with fresh medium every 3 days.

Mucin Secretion Assays, SDS-PAGE and Immunoblotting

LS174T cells (70-80% confluence) were metabolically labeled for 48 hours with 1 μCi/mL [6-^{3}H]glucosamine hydrochloride (40 Ci/mmol; ICN Biomedicals Inc., Irvine, CA). This procedure uniformly labels mucins destined for packaging in secretory granules and consequently represents the preformed mucin pool.[17,18] In dose response experiments, supernatants from cells stimulated with various concentrations of PGs or EP receptor agonists were collected after 12 hours. For time course experiments, labeled cells were washed 3 times with fresh medium then stimulated for 0.5, 1, 3, 6 and 12 hours in medium containing ethanol vehicle, 10 μmol/L PMA, 50 μmol/L forskolin or 250 nmol/L PGE_2. For inhibition studies, cells were pretreated with the adenylate cyclase inhibitor SQ22536 (100 μmol/L) for 30 minutes at 37°C then stimulated with 250 nmol/L PGE_2 for 3 hours in the presence of the inhibitor. In all in vitro experiments, supernatants were collected, cell debris was removed by centrifugation (1500*g* for 5 minutes) and the secreted radiolabeled glycoproteins were precipitated overnight at 4°C by the addition of an equal volume of 10% trichloroacetic acid containing 1% phosphotungstic acid (Sigma Chemical Co.). Precipitated glycoproteins were pelleted by centrifugation (2,000*g* for 5 minutes),

resuspended in a total volume of 5 mL of phosphate-buffered saline following neutralization to pH 7.0 with 0.1 mol/L NaOH. To quantify mucin secretion in response to secretagogues, secreted ^{3}H-labeled mucins were isolated by Sepharose 4B (S4B) column chromatography as follows: equal volumes (0.5 mL) of precipitated glycoproteins from each condition were eluted on a S4B chromatography column as previously described.[19] Fractions (0.5 ml) were collected and aliquots (0.25 mL) from the V_o fractions (fractions 6-10) were dispensed into individual scintillation vials containing 5 mL liquid scintillation fluid and ^{3}H-activity was monitored by liquid scintillation counting. [^{3}H]glucosamine-labeled mucins (30,000 cpm) isolated from the V_o of S4B chromatography were analyzed by SDS-PAGE as described previously.[20] High M_r mucins (V_o) isolated from unstimulated and PGE_2-stimulated cells were analyzed by cesium chloride density gradient centrifugation as described.[19]

Rat Colonic Loop Studies

Male Wistar rats (Charles River, Quebec, Canada) 4-6 weeks old and weighing approximately 260 g were used in this study. Laboratory chow and water were provided ad libitum. Rat colonic loop studies were performed essentially as described.[20,21] Briefly, rats were injected intraperitoneally with 20 μCi of [6-^{3}H]glucosamine hydrochloride (40 Ci/mmol; ICN Biomedicals Inc.) in 0.5 mL of sterile Dulbecco's phosphate buffered saline (PBS, pH 7.2; Life Technologies). After 4 hours, the rats were anesthetized and the abdomen cavity opened. Colonic loops (3 cm long, 2 loops per rat) were surgically tied with black silk suture (Ethicon Inc., Peterborough, Ontario, Canada) to exclude 1 cm distal to the cecum and the entire rectum. The loops were then injected with 0.5 mL of PBS

containing ethanol vehicle, prostaglandin or EP receptor agonist. For S4B chromatography profiles, the entire colon was flushed with 10 mL of warm PBS to remove luminal contents and injected with 1 mL of PBS containing vehicle or agonists. Rats were killed after 2 hours, the entire loop was removed and cut open longitudinally in 1ml warm PBS. The mucosal surface was gently scraped with a glass slide to collect the adherent and secreted mucus. The scrapings were vortexed vigourously (about 5 minutes) then centrifuged at 1,000*g* for 5 minutes to remove cell debris. Secreted ^{3}H-labeled glycoproteins were precipitated and solubilized and mucin secretion quantified by S4B chromatography as described above. The animal experimental procedures were approved by the McGill University Animal Care Committee as recommended by the Canadian Council on Animal Care.

LS174T Membrane Preparation and [^{3}H]PGE$_2$ Binding Assays

For membrane preparations, LS174T cells were grown to 85-90% confluence (3 weeks) in tissue culture flasks (150 cm^2). The cells were washed twice in cold phosphate buffered saline then incubated on ice in hypotonic buffer (15 mmol/L Tris-Hcl, pH 7.4, 1.25 mmol/L $MgCl_2$ and 1.25 mmol/L EDTA) for 10 minutes. Cells were scraped, collected by centrifugation at 1,200*g* for 5 minutes at 4°C, resuspended in sonication buffer (75 mmol/L Tris-HCl, pH 7.4, 25 mmol/L $MgCl_2$, 1 mmol/L EDTA and 1 mmol/L PMSF) then lysed by sonication on ice. Cell debris was removed by centrifugation at 2,000*g* for 5 minutes and the supernatant was then centrifuged at 30,000*g* for 30 minutes at 4°C. The crude membrane pellet was resuspended in storage buffer (20 mmol/L HEPES, pH 7.4, 25 mmol/L $MgCl_2$ and 1 mmol/L EDTA) then snap frozen in liquid nitrogen and

stored at -80°C. For saturation binding studies, replicates of 50 μg of crude membrane proteins were incubated with increasing concentrations of [5,6,8,11,12,14,15(n)-^{3}H]PGE_2 (187 Ci/mmol; Amersham) in binding buffer (10 mmol/L MES (Sigma), pH 6.0, 10 mM $MgCl_2$ and 1 mmol/L EDTA) for 1 hour at room temperature. Non-specific binding was determined in parallel for each concentration using 10 μmol/L unlabeled PGE_2. For competitive binding studies, replicates of 50 μg of membrane proteins were incubated with 3 nmol/L [^{3}H]PGE_2 and increasing concentrations of PGs or EP receptor agonists for 1 hour at room temperature. Following the incubation, 5 mL of ice-cold wash buffer (10 mmol/L MES, pH 6.0 and 0.01% bovine serum albumin) were added to the suspensions and immediately applied to prewetted GF/C filters (Whatman, Maidstone, KY) under rapid-vacuum filtration. The filters were washed twice with 5 mL of ice-cold wash buffer and placed in individual scintillation vials containing 5 mL scintillation fluid for scintillation counting.

$[cAMP]_i$ Measurements in LS174T Cells

LS174T cells grown in 24 well plates (5 X 10^5 cells/well) were incubated with 1 mmol/L of the phosphodiesterase inhibitor (IBMX) for 10 minutes at 37°C then for 15 minutes with IBMX and various PGs or EP receptor agonists at 37°C. For inhibition studies, cells were pretreated with the adenylate cyclase inhibitor SQ22536 (100 μmol/L) and IBMX for 30 minutes at 37°C then stimulated with 250 nmol/L PGE_2 as indicated above. The supernatants were removed and 1 mL of -20°C ethanol was added to each well to extract $[cAMP]_i$. The plates were placed at - 20°C overnight. Cell debris was removed

by centrifugation at 2000*g* for 5 minutes and the ethanol supernatants were dried by vacuum centrifugation. $[cAMP]_i$ was quantified by enzyme immunoassay following the manufacturer's protocol (Cayman).

RT-PCR for EP receptors

Total RNA was extracted from LS174T cells using Trizol reagent (Gibco) following the manufacturer's protocol. Aliquots of 1 μg RNA were treated with DNAse I (Gibco) following the manufacturer's protocol. For RT reactions, 1 μg of the DNAsed RNA was reversed transcribed using oligo(dT) primer (Gibco) in 25 μL reactions. The following primers were used PCR: EP_1 (471 bp), 5'-CGCGCTGCCCATCTTCTCCAT-3' and 5'-CCCAGGCCGATGAAGCACCAC-3'; EP_2 (419 bp), 5'-CAACCTCATCCGCATGCAC-3' and 5'-CTCAAAGGTCAGCCTG-3'; EP_3 (397 bp), 5'-CCATCCCCTCCTCACCTC-3' and 5'-CGATGTGCTCCCAACGCT-3'; EP_4 (434 bp), 5'-TGGTATGTGGGCTGGCTG-3' and 5'-GAGGACGGTGGCGAGAAT-3'. PCR amplifications for EP_2, EP_3 and EP_4 were performed for 40 cycles (39 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute followed by 1 cycle with a final extension of 8 minutes) and contained 1, 1 or 1.5 mmol/L $MgCl_2$, respectively, in 100 μL reaction volumes containing 50 pmol of each primer, 20 μmol/L dNTP, 1 X PCR buffer, 5 U Taq polymerase and 15 μL of cDNA template or 200 ng control cDNA. For EP_1, 40 cycles of PCR amplification were performed (39 cycles of 98°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute followed by 1 cycle with a final extension of 8 minutes) and contained 2.5 U Pwo DNA polymerase (Boehringer Mannheim, Laval, Canada) 50 μmol/L dNTP, 1 x PCR buffer, 2 mmol/L $MgSO_4$ and 15 μL of cDNA template or 200 ng of

control cDNA in a reaction volume of 50 μL. Amplified PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualized by ultraviolet illumination. PCR product identities were confirmed by DNA sequencing (Bio S&T, Lachine, Quebec, Canada).

Data and Statistical Analysis

The EC_{50} is defined as the concentration of drug required to produce a half-maximal response. It was calculated using the software program Prism (Graphpad Software, San Diego, CA). The equilibrium inhibition constants (K_i) were calculated from the equation $K_i = IC_{50}/1+ [radioligand]/K_d$. The IC_{50} is the inhibitory concentration of ligand required to displace 50% of specific binding; [radioligand] is the concentration of radioligand used and K_d is the equilibrium dissociation constant.[22] Data are presented as mean ± SEM and were analyzed by one-way analysis of variance (ANOVA) or Student's *t* test where indicated. A *P* value of <0.05 was considered significant.

Results

EP Receptor mRNA Expression in LS174T Cells

RT-PCR was performed on LS174T mRNA to determine whether they express EP receptor transcripts. Primers pairs were designed to specifically amplify EP_1, EP_2, EP_3 and EP_4. PCR reactions yielded products of expected size from control human EP receptor cDNAs and LS174T mRNA (Figure 1) for EP_2 (419 bp), EP_3 (397 bp) and EP_4 (434 bp). Reverse-transcriptase negative reactions yielded no products. Although we could amplify

the expected size product from human EP_1 cDNA positive control (471 bp), no EP_1 PCR product of expected size could be detected from LS174T mRNA. The identities of the PCR products were confirmed by DNA sequencing.

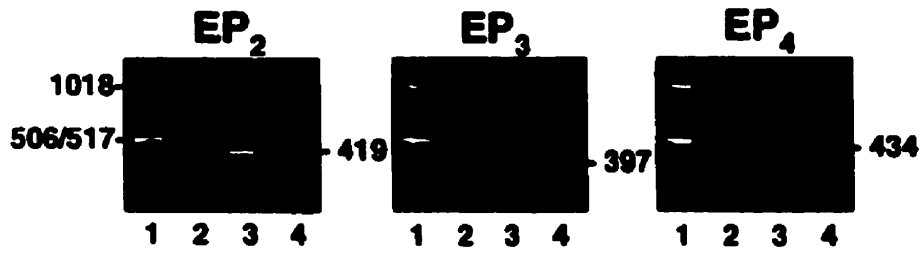


Figure 1. RT-PCR analysis of prostaglandin EP receptor mRNAs expressed by LS174T cells. Total RNA was extracted from LS174T cells and RT-PCR was performed using EP specific primers as described in the "Materials & Methods". Panels show the PCR analysis for EP_2, EP_3 and EP_4, respectively. *Lane 1* in each panel shows the molecular size marker where the 1018 bp and 506/517 bp fragments are indicated on the left. *Lanes 2* and *3* in each panel show reverse transciptase-negative controls and the PCR product of expected size from LS174T mRNA, respectively. *Lane 4* in each panel shows the PCR products from positive control cDNAs: EP_2, 419 bp; EP_3, 397 bp; and EP_4, 434 bp. EP_1 receptor mRNA was not detected in LS17T cells. PCR product identities were confirmed by DNA sequencing.

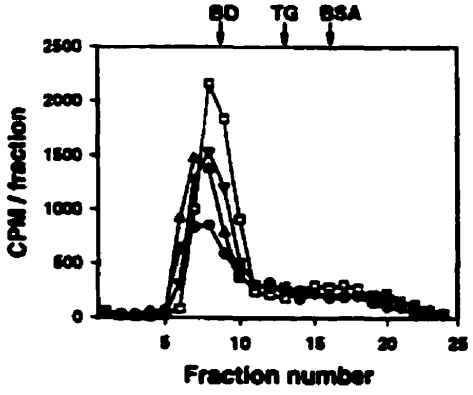
Effect of PGs and EP Receptor Agonists on Mucin Secretion by LS174T Cells

LS174T cells are used as an in vitro model for goblet cell mucin exocytosis.[19] They secrete mucins constitutively and in response to mucin secretagogues.[19,23,24] Secretory mucins are high M_r glycoproteins that elute in the V_o of S4B chromatography and are retained in the stacking gel of SDS-PAGE.[25] As shown in Figure 2A, >60-70% of the ^{3}H-glycoproteins secreted by LS174T cells in response to PGE_2 and other stimuli

partitioned in the S4B V_o. The ^{3}H-glycoproteins isolated from the V_o were retained in the stacking gel of 7% SDS-PAGE (Figure 2B) with no low M_r contaminating proteins. The glycoproteins in the stacking gel were identified as mucins by immunoblot analysis using a rabbit polyclonal antibody raised against CsCl purified LS174T mucins (Figure 2C).[20] In addition, >80% of the S4B V_o ^{3}H-mucins isolated from PGE_2- stimulated cells partitioned in the high-density hexose-rich fractions by CsCl density gradient centrifugation (Figure 2D; density >1.44 g/mL, fractions 6-8). We have previously characterized S4B V_o glycoproteins as purified mucins based on amino acid and carbohydrate compositional analysis and their ability to resist enzymes that degrade proteoglycans.[19] Based on these features, the high M_r glycoproteins that eluted in the S4B V_o were used as a measure for mucin secretion by LS174T cells and rat colon (see below).

Previous studies have shown that PGE_1 and 16,16-dimethyl-PGE_2 stimulates mucin exocytosis in the human intestinal cell lines T84 and HT29-18N2, respectively.[26,27] Accordingly, we determined the effect of various PGs on mucin secretion by LS174T cells and quantified the radiolabeled mucins that accumulated in the medium over 12 hours. As shown in Figure 3A, PGE_2 caused a dose-dependent increase in ^{3}H-labeled mucins released into the medium (EC_{50} of 8.2 ± 1.2 nmol/L). The response to PGE_2 was biphasic: maximum release of ^{3}H-labeled mucins occurred at 250 nmol/L PGE_2 and subsequently declined at higher concentrations. $PGF_{2\alpha}$ also stimulated mucin secretion dose-dependently but maximal stimulation occurred at 1 μmol/L and was overall not as potent as PGE_2 (EC_{50} of 279 ± 37 nmol/L). Iloprost, a stable prostacyclin (PGI_2) receptor

A



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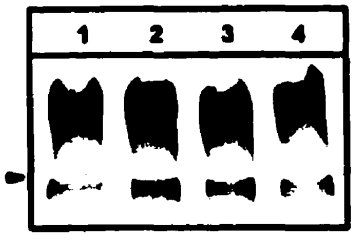

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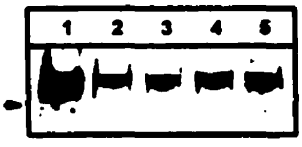

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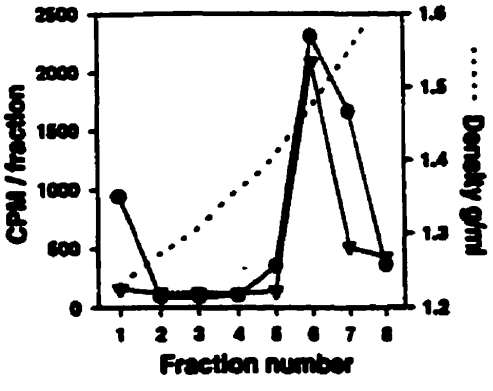

Figure 2. Sepharose 4B chromatography of secreted mucins by LS174T cells. (A) Mucins were metabolically labeled with [^{3}H]glucosamine and isolated by Sepharose 4B chromatography from cells stimulated for 12 hours with media + vehicle (●), 10 μmol/L PMA (□), 50 μmol/L forskolin (Δ) or 100 nmol/L PGE_2 (▼). The cpm in each fraction were monitored by liquid scintillation counting. The V_o (fractions 6-10) of the column is indicated by blue dextran (BD; $M_r > 2 \times 10^6$) and the included fraction are indicated by thyroglobulin (TG; 669,000) and bovine serum albumin (BSA; 67,000). (B) Fluorograph of the pooled [^{3}H]-S4B V_o mucins (30,000 cpm) isolated from above and resolved on 7% SDS-PAGE. Note that the radiolabeled mucins were retained in the stacking gel. The *arrowhead* indicates the border between the stacking and separating gel. (C) Immunoblot of secreted LS174T S4B V_o mucins. CsCl-purified mucins (1 μg) from LS174T cells were used as a control (*lane 1*). The secreted high M_r glycoproteins (50 μg/lane) from unstimulated (*lane 2*), PMA (*lane 3*), forskolin (*lane 4*) or PGE_2 (*lane 5*) stimulated cells collected in the S4B V_o fractions were retained in the stacking gel and were identified as mucins by immunoblotting using an anti-CsCl purified LS174T mucin rabbit immune serum (33). The *arrowhead* indicates the border between the stacking and separating gel. (D) Partitioning of secreted radiolabeled S4B V_o mucins from unstimulated (●) and PGE_2-stimulated (▼) LS174T cells by cesium chloride density gradient centrifugation. Greater than 80% of the recovered radiolabeled mucins were in the high-density fractions (fractions 6-8; density >1.44 g/mL).

(IP) agonist[28] and 13,14-dihydro-15-keto-PGE_2 (a physiologic metabolite of PGE_2) were ineffective at stimulating mucin exocytosis as compared to PGE_2. The rank order of potency of the prostaglandins on mucin secretion was PGE_2 > $PGF_{2\alpha}$ > 13,14-dihydro-15-keto-PGE_2 > iloprost.

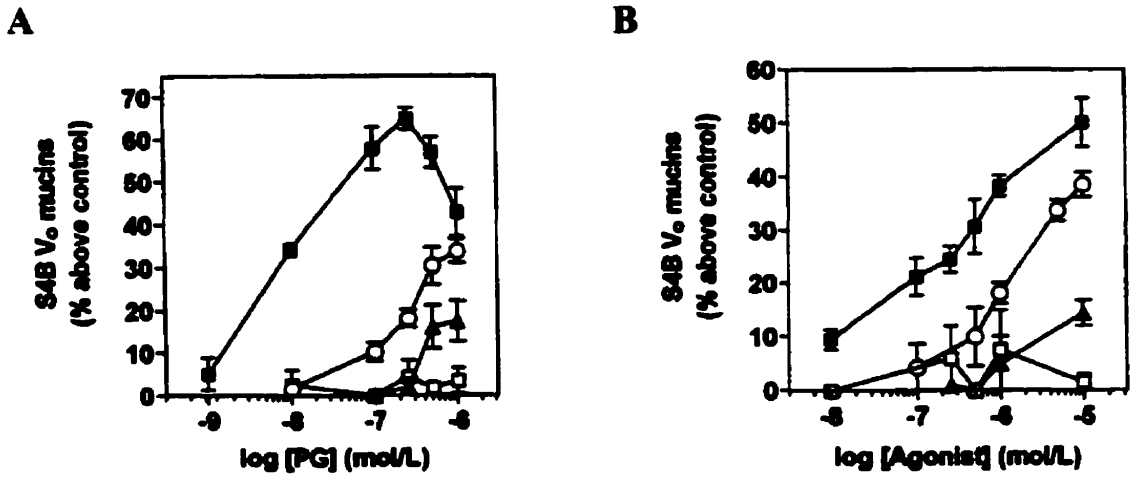

Figure 3. Secretion of [^{3}H]-labeled mucins in response to PGs and EP receptor agonists. LS174T cells (80% confluence) were labeled for 48 hours with [^{3}H]glucosamine then stimulated for 12 hours at 37^{O}C with PGs or EP receptor agonists. Secreted glycoproteins were analyzed by S4B chromatography and each point depicts the percent increase in S4B V_o cpm (mucins) above control. (A) PGs; PGE_2(■), $PGF_{2\alpha}$ (O), iloprost (□), 13,14-dihydro-15-keto-PGE_2 (▲). (B) EP receptor agonists; butaprost (EP_2; ▲), sulprostone (EP_3/EP_1; □); M&B28767 (EP_3/EP_4; O), 1-OH-PGE_1 (EP_4; ■). Data represent the means ± SEM of three to five experiments.

As PGE_2 coupling to EP receptors is necessary to exert its biological effect, we next determined which of the four EP receptor subtypes was linked to mucin exocytosis by studying the effect of EP receptor agonists on mucin secretion. Since increases in $[Ca^{2+}]_i$ and $[cAMP]_i$ can evoke mucin exocytosis, we hypothesized that the EP_1 and EP_2/EP_4

receptors may be linked to mucin secretion. Accordingly, we studied the effect of EP receptor agonists on mucin exocytosis by LS174T cells (Figure 3B). To determine if the EP_4 receptor is coupled to mucin secretion, cells were stimulated with the EP_4 and EP_3/EP_4 receptor agonists 1-OH-PGE_1 and M&B28767, respectively.[4,6,29,30] 1-OH-PGE_1 caused a dose-dependent increase in ^{3}H-labeled mucins secreted into the medium with maximal secretion occurring at 10 μmol/L (EC_{50} of 362 ± 154 nmol/L). M&B28767- stimulated mucin secretion (EC_{50} of 1617 ± 130 nM) but was not as potent as 1-OH-PGE_1 indicating that perhaps it is a weak activator of the EP_4 subtype. In contrast, the IP/EP_1 agonist iloprost[28] and the EP_3/EP_1 agonist sulprostone[31] were virtually ineffective at stimulating mucin release when compared to PGE_2 and thus, excluded both EP_1 and EP_3 in PGE_2-mediated mucin secretion. Butaprost, an EP_2 selective agonist most commonly used to distinguish between EP_2 and EP_4 signaling,[32] caused only a slight increase at 10 μmol/L. The rank order of potency for the EP receptor agonists was 1-OH-PGE_1 (EP_4) > M&B28767 (EP_3/EP_4) > butaprost (EP_2) > sulprostone (EP_3/EP_1).

We next determined the time dependency of PGE_2 on mucin secretion. For these studies, PMA was used as a positive control as it stimulates protein kinase C-dependent mucin release[26] whereas forskolin, a non-receptor mediated $[cAMP]_i$-elevating agent was used to determine $[cAMP]_i$-dependent mucin secretion. As shown in Figure 4, negligible secretion occurred during the initial 30 minutes following addition of the mucin secretagogues. The onset of secretagogue-induced exocytosis occurred between 30 and 60 minutes. PMA (10 μmol/L) evoked a pronounced secretory response at 1 hour (>60% of

maximal secretion), where maximal secretion occurred at 3 hours and stabilized thereafter. In contrast, mucin secretion in response to PGE_2 (250 nmol/L) and forskolin (50 μmol/L) paralleled each other and was less pronounced after 1 hour (less than 35 and 45% of maximal secretion, respectively), with maximal secretion occurring after 6 hours. These results highlight the differences in secretory responses to secretagogues linked to different intracellular signaling pathways.

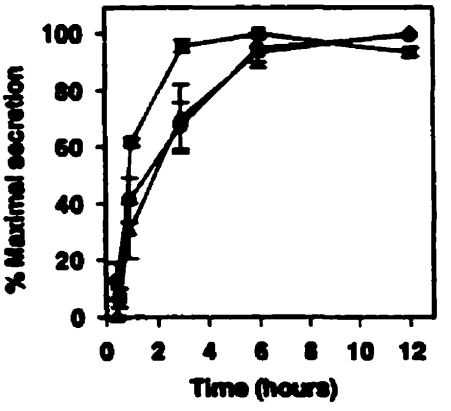

Figure 4. Time course of [^{3}H]glucosamine-labeled mucin secretion by LS174T cells stimulated with secretagogues. Cells were stimulated for 0.5, 1, 3, 6 and 12 hours with 10 μmol/L PMA (■), 50 μmol/L forskolin (●), or 250 nmol/L PGE_2 (▲), and the secreted mucins that accumulated in the supernatants were quantified by S4B chromatography as described in "Materials & Methods". Each point depicts the percent of maximal secretion above control. Data represent the means ± SEM of three experiments.

Effect of PGs and EP Receptor Agonists on Mucin Secretion in Rat Colon

To determine if signaling via the EP_4 receptor also stimulates mucin secretion in vivo, the effects of PGE_2, its inactive metabolite 13,14-dihydro-15-keto-PGE_2 and various EP receptor agonists were tested in rat colonic loop studies. As shown in Figure

5A, loops inoculated with 5 μmol/L PGE_2 or the EP_4 receptor agonist 1-OH-PGE_1 stimulated 64.0 ± 15.7% and 53.5 ± 11.1% increases in high M_r V_o mucins respectively, as compared to control loops injected with vehicle alone ($P< 0.05$). Futhermore, >85% of the ^{3}H-glyoproteins released in response to PGE_2 and 1-OH-PGE_1 were low M_r glycoproteins (Figure 5B), suggesting that the agonists have mucin and non mucin secretagogue activity. In contrast, an equimolar concentration (5 μmol/L) of the metabolite 13,14-dihydro-15-keto-PGE_2, the EP_2 agonist butaprost (10 μmol/L) and the EP_3/EP_1 agonist sulprostone (5 μmol/L) were ineffective at stimulating mucin release (Figure 5A). From these experiments we conclude that the EP_4 receptor is linked to mucin exocytosis in rat colon which corroborates the data in the human colonic cell line LS174T.

EP Receptor Characterization in LS174T Cell Membranes

To characterize the interaction between PGE_2 and the EP_4 receptor, the kinetics of [^{3}H]PGE_2 binding to LS174T cell membranes was determined. [^{3}H]PGE_2 specific binding to cell membranes was dose-dependent and displayed saturable binding kinetics. Computer assisted non-linear regression analysis of binding data using the LIGAND program[33] revealed a high affinity binding site with a K_d of 0.27 ± 0.08 nmol/L and a B_{max} of 52.9 ± 7.8 fmol/mg. In subsequent competition binding studies, a concentration of 3 nmol/L [^{3}H]PGE_2 was used. Unlabeled PGs competitively inhibited the specific binding of [^{3}H]PGE_2 (Figure 6A) with a rank order of affinity PGE_2 > $PGF_{2\alpha}$ > iloprost > 13,14-

dihydro-15-keto-PGE_2. Their K_i values were 0.4, 30, 77 and 124 nmol/L, respectively. The EP receptor agonists competitively inhibited $[^3H]PGE_2$ specific binding

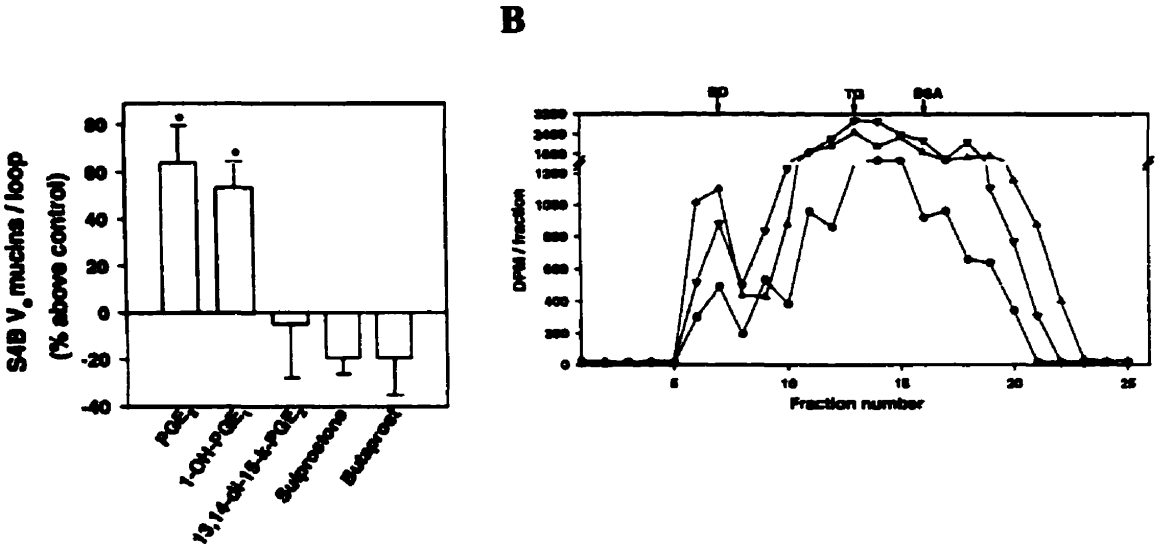

Figure 5. Secretion of ^3H-labeled mucins and nonmucin glycoproteins in rat colon. Rats were injected intraperitoneally with $[^3H]$glucosamine for 4 hours to metabolically labeled the mucins. Colonic loops were made and injected with ethanol vehicle or 5 μmol/L of either PGE_2, 1-OH-PGE_1, 13,14-dihydro-15-keto-PGE_2, sulprostone or 10 μmol/L butaprost for 2 hours. Secreted mucins were isolated and quantified by S4B chromatography and liquid scintillation counting. (A) PGE_2 and the EP_4 agonist 1-OH-PGE_1 caused a significant ($P<0.05$ compared to vehicle control by Student's *t*-test) increase in mucin secretion (measured as S4B V_o mucins) whereas the other agonists had no effect (n=3 or 4 loops per condition except butaprost where n=2). (B) S4B chromatography profiles of ^3H-labeled glycoproteins secreted in response to vehicle (●), 5 μmol/L PGE_2 (▼), or 5 μmol/L 1-OH-PGE_1 (Δ) in rat colon. The V_o (fractions 6-8) of the column is indicated by blue dextran (BD; $M_r > 2 \times 10^6$) and the included fraction are indicated by thyroglobulin (TG; 669,000) and bovine serum albumin (BSA; 67,000).

(Figure 6B) with the rank order of affinity M&B28767 (EP_3/EP_4) > 1-OH-PGE_1 (EP_4) > sulprostone (EP_3/EP_1) > butaprost (EP_2). Their K_i values were 12.5, 307, >1000 and >1000 nmol/L, respectively. Reversibility of binding with unlabeled PGE_2, especially at lower concentrations (<100 nmol/L) (Figure 6A), clearly demonstrated specificity of

binding to an EP receptor. Furthermore, the rank order of affinity for the EP receptor agonists is consistent with the high affinity site being of the EP_4 subtype. These results show that PGE_2 binds to a specific high affinity site on LS174T cells which displays a binding profile congruous with the EP_4 receptor.

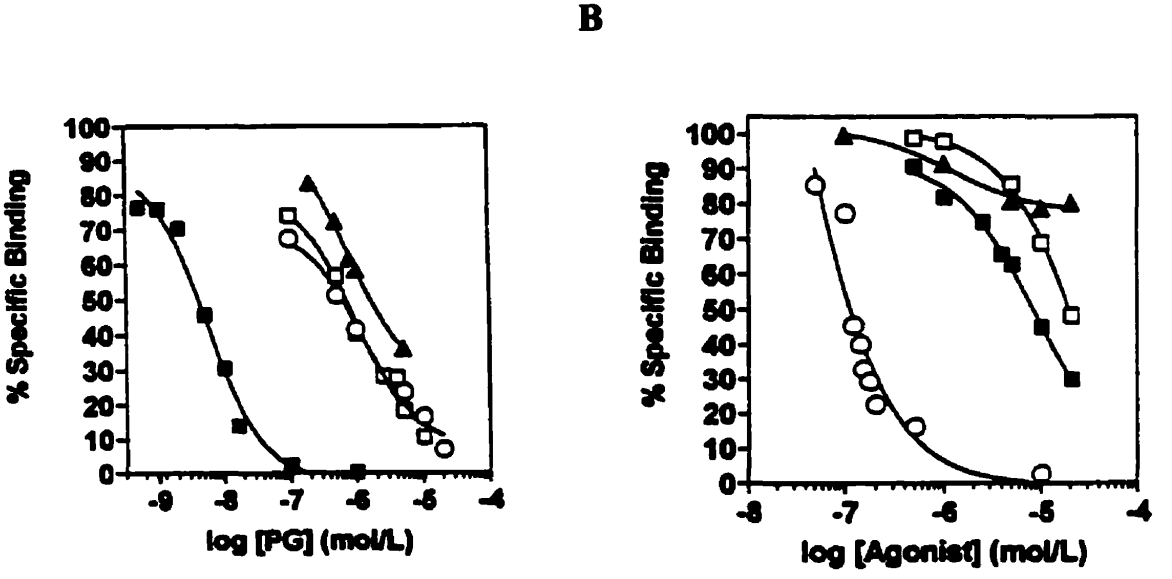

Figure 6. Competition of $[^3H]PGE_2$ specific binding to LS174T cell membranes by PGs and EP receptor agonists. Membranes (50 µg) from LS174T cells were incubated with 3 nmol/L $[^3H]PGE_2$ and various concentrations of PGs and EP receptor agonists for 1 hour at room temperature. The suspensions were applied to filters by rapid vacuum filtration and binding was quantified by liquid scintillation counting. Specific binding was determined in parallel with 10 µmol/L PGE_2. (A) PGs: PGE_2 (■), $PGF_{2\alpha}$ (O), iloprost (□), 13,14-dihydro-15-keto-PGE_2 (▲). (B) EP receptor agonists: butaprost (EP_2; ▲), sulprostone (EP_3/EP_1; □), M&B28767 (EP_3/EP_4; O), 1-OH-PGE_1 (EP_4; ■). The data represent the mean of duplicate determinations from an experiment that was repeated 3 times with similar results.

Ligand Binding to EP_4 Increases $[cAMP]_i$ in LS174T Cells

The EP_4 receptor is coupled to increases in $[cAMP]_i$ through a $G_{\alpha s}$ protein.[6] As

PGE_2 interacts with EP_4 to stimulate mucin exocytosis, we anticipated a concomitant rise in $[cAMP]_i$ would occur in PGE_2- and EP_4 agonist-stimulated LS147T cells. As expected, the adenylate cyclase activator forskolin (50 μmol/L) stimulated significant $[cAMP]_i$ production, approximately 45-fold increase above control ($P< 0.05$, one-way ANOVA) (Table 1). Similarly, PGE_2 and the EP_4 agonists 1-OH-PGE_1 and M&B28767 caused

Table 1. $[cAMP]_i$ production by LS174T cells following stimulation with forskolin or EP receptor agonists

Stimulus	Concentration (nmol/L)	pmol $[cAMP]_i$/5 x 10^5 cells[a]
Control		5.6 ± 1.0
Forskolin	5.0 x 10^4	260 ± 21*
PGE_2	10	52.5 ± 6.3*
	100	163 ± 13*
	250	198 ± 6.3*
1-OH-PGE_1 (EP_4)	100	56.5 ± 8.1*
	250	106 ± 14*
	500	168 ± 12*
M&B28767 (EP_3/EP_4)	1.0 x 10^3	7.9 ± 0.5
	1.0 x 10^4	34 ± 3.2*
	2.0 x 10^4	52.3 ± 4.3*
Butaprost (EP_2)	1.0 x 10^3	9.2 ± 1.0*
Sulprostone (EP_3/EP_1)	1.0 x 10^3	0.7 ± 0.4*

[a]Cells were pretreated with 1 mmol/L of IBMX for 10 minutes and then stimulated for 15 minutes with IBMX plus various PGs or EP receptor agonists at 37°C. $[cAMP]_i$ was extracted in ethanol at -20°C overnight. Cell debris was removed by centrifugation at 2000*g* for 5 minutes and the ethanol supernatants were dried by vacuum centrifugation. $[cAMP]_i$ was quantified by enzyme immunoassay following the manufacturer's protocol. *Values represent the mean ± SEM of the results of three experiments. $P< 0.05$, one-way ANOVA.

dose-dependent increases in $[cAMP]_i$ where maximal production occurred at 250 nmol/L

(35-fold increase), 500 nmol/L (30-fold increase) and 20 μmol/L (9-fold increase), respectively. In contrast, the EP_2 agonist butaprost (1 μmol/L; 1.6-fold increase) caused only a minimal increase in $[cAMP]_i$ whereas sulprostone caused a slight but significant decrease in $[cAMP]_i$ production (Table 1). To verify that PGE_2-mediated mucin secretion was dependent on $[cAMP]_i$, cells were treated with the adenylate cyclase inhibitor SQ22536 (100 μmol/L) prior to stimulation with 250 nmol/L PGE_2. The inhibitor reduced $[cAMP]_i$ by 27% (147.5 ± 14.9 versus homologous control 203.3 ± 20.2 pmol/5 x 10^5, $P< 0.05$, one-way ANOVA) in pretreated cells when compared to cells stimulated with PGE_2 alone. Furthermore, PGE_2-mediated mucin exocytosis was inhibited by 46.0 ± 8.9% when compared to untreated PGE_2-stimulated cells. Taken together, these results clearly demonstrate that PGE_2 interacts at the EP_4 but not the EP_2 receptor to induce $[cAMP]_i$-dependent mucin exocytosis in LS174T cells.

Discussion

The regulation of mucin secretion during normal and inflammatory states plays a critical role in the maintenance of the intestinal epithelial barrier. Mucins secreted in response to inflammatory mediators such as PGE_2 may play a concerted role in the restitution of damaged intestinal epithelia. Indeed, recent studies suggest that prostaglandins may be important mediators of host tissue repair during inflammatory states.[13,34] Goblet cells may respond to PGE_2 by releasing copious amounts of mucins to

protect the damaged epithelium. Although past efforts have shown that PGE_2 analogues induce mucin secretion by colonic cells,[26,27] the specific PGE_2 receptor(s) involved in mediating mucin exocytosis was not shown. Identifying the receptor-coupled signaling pathway is central to our comprehension of how mucin exocytosis is regulated during inflammatory states. It was therefore our goal to describe in detail the receptor coupling events involved in PGE_2-mediated mucin exocytosis.

Stimulation of LS174T cells with PGE_2 caused a dose-dependent increase in radiolabeled mucins released into the medium. Of the PGs tested, PGE_2 was the most potent and effective at low concentrations. Although $PGF_{2\alpha}$ stimulated mucin exocytosis, its activity may in part be due to cross-reactivity at the EP_4 receptor since it displaced the binding of $[^3H]PGE_2$ in receptor binding studies and stimulated a small but significant increase (4-fold above control) in $[cAMP]_i$. The IP/EP_1 receptor agonist iloprost was ineffective at stimulating mucin exocytosis and therefore excluded IP receptor involvement. We used the physiologic metabolite of PGE_2, 13,14-dihydro-15-keto-PGE_2, as a negative control in mucin secretion experiments and it was completely inactive at an equimolar dose of PGE_2 causing maximal mucin output (Figure 3A).

Stimulation with higher concentrations (>250 nmol/L) of PGE_2 caused a sub-optimal accumulation of radiolabeled mucins in the culture medium. Other studies have reported biphasic secretory responses to PGE_2. For example, low doses of PGE_2 inhibited acid secretion by gastric parietal cells whereas high doses stimulated secretion.[9] Parietal cells, much like LS174T cells, were shown to express both EP_3 and EP_4 mRNAs and

respond biphasically to PGE_2.[9] Furthermore, PGE_2 stimulated histamine release by ECL cells biphasically.[35] Histamine, like mucins, is packaged and stored in granules and is released by the process of compound exocytosis. The biphasic response at higher concentrations of PGE_2 may perhaps be explained by receptor desensitization or modulation of $[cAMP]_i$ production via feedback inhibition of adenylate cyclase.[36]

Analysis of EP receptor distribution in mouse gastrointestinal tissues demonstrated that the EP_4 receptor appeared to be expressed in mucus-producing cells of the stomach.[9] In our studies, we used EP receptor agonists to demonstrate that the EP_4 receptor was expressed by LS174T cells and linked to mucin exocytosis. The EP_4 receptor agonist 1-OH-PGE_1 and the EP_3/EP_4 agonist M&B28767 both stimulated mucin exocytosis dose-dependently, implicating EP_4 as the EP receptor linked to mucin exocytosis. Iloprost (IP/EP_1), butaprost (EP_2) and sulprostone (EP_3/EP_1) were relatively ineffective at stimulating mucin secretion and thus excluded the EP_1, EP_2 and EP_3 receptors, respectively. Although a novel prostaglandin receptor positively coupled to increases in $[cAMP]_i$ has been reported in the Jurkat T-cell line, it was insensitive to 1-OH-PGE_1 up to 100 μmol/L.[37] This is in stark contrast to our findings in that 1-OH-PGE_1 was effective at concentrations as low as 100 nmol/L and thus, rules out the possibility of signaling via this novel receptor. RT-PCR and DNA sequencing analysis confirmed that LS174T cells express EP_4 receptor mRNA. In addition, EP_2, EP_3, but not EP_1 receptor transcripts were detected. Other cell types including B cells, T cells and macrophages express multiple EP receptor mRNAs.[38-40] Although we detected EP_2 mRNA by RT-PCR, the EP_2-selective

agonist butaprost caused only slight mucin output and $[cAMP]_i$ production indicating that EP_2 receptors contribute minimally to the secretory response in these cells. Furthermore, it is not surprising that EP_3 is not involved in mucin exocytosis as it is coupled to $G_{\alpha i}$ and inhibits the formation of $[cAMP]_i$. Several reports have demonstrated EP_3 mRNA splice variants coupled positively to adenylate cyclase and $[Ca^{2+}]_i$.[7,8,41,42] This would not appear to be the case in LS174T cells as sulprostone did not stimulate mucin exocytosis nor elevate $[cAMP]_i$. Interestingly, sulprostone caused net fluid secretion in rat ileum by inhibiting unidirectional absorption of ^{22}Na whereas PGE_2 caused net fluid secretion by provoking ^{22}Na efflux.[43] This suggests that intestinal ionic balance and mucin secretion may be modulated by different EP receptors.

Studies in rat colonic loops confirmed that PGE_2 and 1-OH-PGE_1 (5 μmol/L) were potent mucin secretagogues. A similar concentration of PGE_2 administered intraluminally in rat and human colon caused marked water and electrolyte secretion.[44,45] In contrast, the PGE_2 metabolite 13,14-dihydro-15-keto-PGE_2 did not stimulate increases in mucin output nor did the EP receptor agonists butaprost and sulprostone. Taken together, these data demonstrate that signaling via the EP_4 receptor stimulates mucin exocytosis which may work in an analogous fashion in the human colon.

Receptor binding studies demonstrated that the high affinity PGE_2 binding site in LS174T cell membranes displayed a binding profile consistent with the EP_4 receptor. The K_d for the high affinity binding site in LS174T cells was slightly lower than the K_d for the cloned human EP_4 receptor (0.27 nmol/L as compared to the reported K_d = 1 nmol/L).[6]

However, a PGE_2 receptor linked to increases in $[cAMP]_i$ with a similar K_d was observed in isolated rabbit colonic crypt cells.[46] Activation of the EP_4 receptor requires the displacement of the $G_{\alpha s}$ protein and subsequent interaction with adenylate cyclase to stimulate $[cAMP]_i$ production. PGE_2 and the EP_4 agonists 1-OH-PGE_1 and M&B28767 caused dose-dependent increases in $[cAMP]_i$ in LS174T cells. Both EP_4 agonists were less effective at stimulating $[cAMP]_i$ production (and consequently mucin secretion) when compared to PGE_2. It would appear that M&B2867 is a partial agonist at the EP_4 receptor, resulting in only approximately 26% of the $[cAMP]_i$ response to PGE_2 and substantiating that it was also less potent at stimulating mucin exocytosis. In cells co-expressing EP_3/EP_4, M&B28767 may at higher concentrations activate both $G_{\alpha i}$ and $G_{\alpha s}$ resulting in the modulation of $[cAMP]_i$ production and thus explaining its lower agonistic effect on mucin secretion. In contrast to these findings, the EP_3/EP_1 agonist sulprostone actually caused a slight decrease in basal $[cAMP]_i$ production. Although EP_3 transcripts were detected, radio-receptor binding studies may not be sensitive enough to detect low receptor numbers.

Using the adenylate cyclase inhibitor SQ22536, we demonstrated the dependence of PGE_2-mediated mucin exocytosis on $[cAMP]_i$. Almost nothing is known about the cellular effectors (exocytosis machinery) activated by second messengers (i.e. $[cAMP]_i$) and involved in mucin exocytosis. Whether distinct signaling pathways utilize the same effectors to mobilize stored mucin granules is unknown. Our time course experiments revealed that mucin exocytosis did not occur immediately after addition of secretagogues but instead required between 30 and 60 minutes before the exocytosis machinery was

activated. Moreover, induction of different signaling pathways resulted in differential secretory responses. The protein kinase C activator PMA induced a more transient and robust secretory response that remained active for approximately 3 hours. In contrast, forskolin and PGE_2 evoked a more gradual activation of exocytosis that was sustained for approximately 6 hours. This observation highlights the differences in secretory responses to secretagogues evoking different signaling pathways.

The main secretory mucin in the human colon is MUC2.[47] Although the *MUC3* gene is expressed in intestinal tissues, it is unclear whether MUC3 is a secretory mucin.[48,49] The *MUC3* gene is not highly expressed in the colon and MUC3 may not be a major component of colonic mucus.[49] LS174T cells express the intestinal mucin genes *MUC2* and *MUC3* but secrete predominantly MUC2.[50,51] Although we cannot exclude that the total mucins secreted in response to PGE_2 stimulation may contain other mucins, the majority of the secreted mucins are most likely derived from *MUC2*. This study has unraveled for the first time the mechanism whereby PGE_2 coupling to the EP_4 receptor on colonic epithelial cells evokes mucin exocytosis, an important constituent for epithelial barrier function. Elucidation of the mode of action of PGE_2 on intestinal mucin secretion may aid in alleviating symptoms of disorders where altered mucin secretion plays a role in pathogenesis and disease states. The development of highly specific EP receptor agonists/antagonists will undoubtedly facilitate this process.

References

1. Smith WL. The eicosanoids and their biochemical mechanisms of action. Biochem J

1989; 259:315-324.

2. Phipps RP, Stein SH, Roper RL. A new view of prostaglandin E regulation of the immune response. Immunol Today 1991;12:349-352.

3. Funk C, Furci L, FitzGerald G, Grygorczyk R, Rochette C, Bayne MA, Abramovitz M, Adam M, Metters KM. Cloning and expression of a cDNA for the human prostaglandin E receptor EP_1 subtype. J Biol Chem 1993;268:26767-26772.

4. Regan JW, Bailey TJ, Pepperl DJ, Pierce KL, Bogardus AM, Donello JE, Fairbairn CE, Kedzie KM, Woodward DF, Gil DW. Cloning of a novel human prostaglandin receptor with characteristics of the pharmacologically defined EP_2 subtype. Mol Pharmacol 1994;46:213-220.

5. An S, Yang J, Xia M, Goetzl EJ. Cloning and expression of the EP_2 subtype of human receptors for prostaglandin E_2. Biochem Biophys Res Commun 1993;197:263-270.

6. Bastien L, Sawyer N, Grygorczyk R, Metters KM, Adam M. Cloning, functional expression, and characterization of the human prostaglandin E_2 receptor EP_2 subtype. J Biol Chem 1994;269:11873-11877.

7. Adam M, Boie Y, Rushmore TH, Müller G, Bastien L, McKee KT, Metters KM, Abramovitz M. Cloning and expression of the three isoforms of the human EP_3 prostanoid receptor. FEBS Letters 1994;338:170-174.

8. Regan JW, Bailey TJ, Donello JE, Pierce KL, Pepperl DJ, Zhang D, Kedzie KM, Fairbairn CE, Bogardus AM, Woodward DF, Gil DW. Molecular cloning and expression of human EP_3 receptors: evidence of three variants with differing carboxyl termini. Br J Pharmacol 1994;112:377-385.

9. Ding M, Kinoshita Y, Kishi K, Nakata H, Hassan S, Kawanami C, Sugimoto Y, Katsuyama M, Negishi M, Narumiya S, Ichikawa A, Chiba T. Distribution of prostaglandin E receptors in the rat gastrointestinal tract. Prostaglandins 1997;53:199-216.

10. Morimoto K, Sugimoto Y, Katsuyama M, Oida H, Tsuboi K, Kishi K, Kinoshita Y, Negishi M, Chiba T, Narumiya S, Ichikawa A. Cellular localization of mRNAs for prostaglandin E receptor subtypes in mouse gastrointestinal tract. Am J Physiol 1997;272:G681-G687.

11. Takeuchi K, Yagi K, Kato S, Ukawa H. Roles of prostaglandin E-receptor subtypes in gastric and duodenal bicarbonate secretion in rats. Gastroenterology 1997;113:1553-1559.

12. Sakai H, Kumano E, Ikari A, Takeguchi N. A gastric housekeeping Cl^- channel activated via prostaglandin EP_3 receptor-mediated Ca^{2+}/Nitric oxide/cGMP pathway. J Biol Chem 1995;270:18781-18785.

13. Robert A, Nezamis JE, Phillips JP. Effect of prostaglandin E_1 on gastric secretion and ulcer formation in the rat. Gastroenterology 1979;55:481-487.

14. Eberhart CE, Duboi RN. Eicosanoids and the gastrointestinal tract. Gastroenterology 1995;109:285-301.

15. Blikslager AT, Roberts MC, Rhoads JM, Argenzio RA. Prostaglandins I_2 and E_2 have a synergistic role in rescuing epithelial barrier function in porcine ileum. J Clin Invest 1997;100:1928-1933.

16. Forstner G. Signal transduction, packaging and secretion of mucins. Annu Rev Physiol 1995;57:585-605.

17. Kim KC, Nassiri J, Brody JS. Mechanisms of airway goblet cell mucin release: studies with cultured tracheal surface epithelial cells. Am J Resp Cell Mol Biol 1989;1:137-143.

18. Augeron C, Voisin T, Maoret JJ, Berthon B, Laburthe M, Laboisse CL. Neurotensin and neuromedin N stimulate mucin output from human goblet cells (Cl.16E) via neurotensin receptors. Am J Physiol 1992;262:G470-476.

19. Belley A, Keller K, Grove J, Chadee K. Interaction of LS174T human colon cancer mucins with *Entamoeba histolytica*: an in vitro model for colonic disease. Gastroenterology 1996;111:1484-1492.

20. Tse S-K, Chadee K. Biochemical characterization of rat colonic mucins secreted in response to *Entamoeba histolytica*. Infect Immun 1992;60:1603-1612.

21. Chadee K, Keller K, Forstner J, Innes DJ, Ravdin JI. Mucin and nonmucin

secretagogue activity of *Entamoeba histolytica* and cholera toxin in rat colon. Gastroenterology 1991;100:986-997.

22. Cheng Y, Prusoff WH. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction. Biochem Pharm 1973;22:3099-3108.

23. Siddiqui B, Byrd JC, Fearney FJ, Kim YS. Comparison of metabolically labeled mucins of LS174T human colon cancer cells in tissue culture and xenograft. Tumor Biol 1989;10:83-94.

24. Keller K, Olivier M, Chadee K. The fast release of mucin secretion from human colonic cells induced by *Entamoeba histolytica* is dependent on contact and protein kinase C activation. Arch Med Res 1992;23:217-221.

25. Podolsky DK, Isselbacher KJ. Composition of human colonic mucin: selective alteration in inflammatory bowel disease. J Clin Invest 1983;72:142-153.

26. McCool DJ, Marcon MA, Forstner JF, Forstner GG. The T84 human colonic adenocarcinoma cell line produces mucin in culture and releases it in response to various secretagogues. Biochem J 1990;267:491-500.

27. Phillips TE, Stanley CM, Wilson J. The effect of 16,16-dimethyl prostaglandin E_2 on proliferation of an intestinal goblet cell line and its synthesis and secretion of mucin glycoproteins. Pros Leuk Essen Fatty Acids 1993;48:423-428.

28. Dong YJ, Jones RL, Wilson NH. Prostaglandin E receptor subtypes in smooth muscle: agonist activities of stable prostacyclin analogues. Br J Pharmacol 1986;87:97-107.

29. Kiriyama M, Ushikubi F, Kobayashi T, Hirata M, Sugimoto Y, Narumiya S. Ligand binding specificities of the eight types and subtypes of the mouse prostanoid receptors expressed in Chinese hamster ovary cells. Br J Pharmacol 1997;122:217-24.

30. Boie Y, Stocco R, Sawyer N, Slipetz DM, Ungrin MD, Neuschäfer-Rube F, Püschel GP, Metters KM, Abramovitz M. Molecular cloning and characterization of the four rat prostaglandin E_2 prostanoid receptor subtypes. Eur J Pharmacol 1997;340:227-241.

31. Coleman RA, Kennedy I, Sheldrick RL. New evidence with selective agonists and antagonists for the subclassification of PGE_2-sensitive (EP) receptors. Adv Prostaglandin Thromboxane Leukotriene Res 1987;17:467-470.

32.Gardiner PJ. Characterization of prostanoid relaxant/inhibitory receptors (psi) using a highly selective agonist, TR4979. Br J Pharmacol 1986;87:45-56.

33. Munson PJ, Rodbard D. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 1980;107:220-239.

34. Reuter BK, Asfaha S, Buret A, Sharkey KA, Wallace JL. Exacerbation of inflammation-associated colonic injury in rat through inhibition of cyclooxygenase-2. J Clin Invest 1996;98:2076-2085.

35. Naribayashi-Inomoto Y, Ding M, Nakata H, Narumiya S, Sugimoto Y, Honda A, Ichikawa A, Chiba T, Kinoshita Y. Copresence of prostaglandin EP_2 and EP_3 receptors on gastric enterochromaffin like cell carcinoid in African rodents. Gastroenterology 1995;109:341-347.

36. Iwami G, Kawabe J-I, Ebina T, Cannon PJ, Homcy CJ, Ishikawa YJ. Regulation of adenylyl cyclase by protein kinase A. Biol Chem 1995;270:12481-12484

37. Devries GW, Guarino P, McLaughlin A, Chen J, Andrews S, Woodward DF. An EP receptor with a novel pharmacological profile in the T-cell line Jurkat. Br J Pharmacol 1995;115:1231-1234.

38. Fedyk ER, Phipps RP. Prostaglandin E_2 receptors of the EP_2 and EP_4 subtypes regulate activation and differentiation of mouse B lymphocytes to IgE-secreting cells. Proc Natl Sci USA 1996;93:10978-10983.

39. Zeng L, An S, Goetzl EJ. Regulation of expression of matrix metalloproteinase-9 in early human T cells of the HSB.2 cultured line by the EP_3 subtype of prostaglandin E_2 receptor. J Biol Chem 1996;271:27744-27750.

40. Zeng L, An S, Goetzl EJ. Independent down-regulation of EP_2 and EP_3 subtypes of the prostaglandin E_2 receptors on U937 human monocytic cells. Immunology 1995;86:620-628.

41. Namba T, Sugimoto Y, Negishi M, Irie A, Ushikubi F, Kakizuka A, Ito S, Ichikawa A, Narumiya S. Alternative splicing of C-terminal tail of prostaglandin E receptor subtype EP_3 determines G-protein specificity. Nature 1993;365:166-170.

42. Yang J, Xia M, Goetzl EJ, An S. Cloning and expression of the EP_3-subtype of human receptors for prostaglandin E_2. Biochem Biophys Res Commun 1994;198:999-1006.

43. Bunce KT, Clayton NM, Knight M, Sheppard DN, Spraggs CF. Differential effects of prostaglandins on unidirectional absorption and secretion in rat ileum. Gastroenterology 1987;92:1332P.

44. Synnerstad I, Holm L. Prostaglandin E_2 and aggressive factors increase gland luminal pressure in the rat gastric mucosa in vivo. Gastroenterology 1998;114:1276-1286.

45. Roze C, Bruley Des Varannes S, Shi G, Geneve J, Galmiche JP. Inhibition of prostaglandin-induced intestinal secretion by igmesine in healthy volunteers. Gastroenterology 1998;115:591-596.

46. Homaidan FR, Zhao L, Burakoff R. Characterization of PGE_2 receptors in isolated colonic crypt cells. Am J Physiol 1995;268:G270-G275.

47. Tytgat KMAJ, Buller HA, Opdam FJM, Kim YS, Einerhand AWC, Dekker J. Biosynthesis of human colonic mucin: MUC2 is the prominent secretory mucin.

Gastroenterology 1994;107: 1352-1363.

48. Chang S-K, Dohrman FA, Basbaum CB, Ho SB, Tsuda T, Toribara NW, Gum Jr. JR, Kim YS. Localization of mucin (MUC2 and MUC3) messenger RNA and peptide expression in human normal intestine and colon cancer. Gastroenterology 1994;107:28-36.

49. Gum Jr. JR, Ho JJL, Pratt WS, Hicks JW, Hill AS, Vinall LE, Roberton AM, Swallow DM, Kim YS. MUC3 human intestinal mucin. J Biol Chem 1997;272:26678-26686.

50. Niv Y, Byrd JC, Ho SB, Dahiya R, Kim YS. Mucin synthesis and secretion in relation to spontaneous differentiation of colon cancer cell in vitro. Int J Cancer 1992;50:147-152.

51. McGuckin MA, Devine PL, Ward BG. Early steps in the biosynthesis of MUC2 epithelial mucin in colon cancer cells. Biochem Cell Biol 1996;74:87-93.

Section III: General Discussion

The small and large intestinal epithelium constitute the largest surface area of the body exposed to antigens. To protect the intestinal epithelium from noxious agents and prevent damage to underlying tissues, a number of defence mechanisms have evolved (1). Bicarbonate is secreted in the duodenum to neutralize stomach acid that has entered via the pyloric sphincter. Tight junctions between epithelial cells limit the entry of chemicals and microorganisms through the paracellular route. Perturbation of ionic balance causes watery diarrhea which flushes organisms from the intestinal lumen. Secretory mucins polymerize into a viscous lubricating gel that acts as a physical barrier to luminal irritants and protects the epithelium from attachment by enteric pathogens (2). These protective mechanisms are constituents of the epithelial barrier function. Combined with the immunologic component which includes secretory IgA, intra-epithelial lymphocytes and myeloid cells of the lamina propria, the intestines are well adapted to the environmental stresses they encounter. Despite these formidable defenses, enteric pathogens can overcome these barriers, perhaps by exploiting them to induce pathophysiologic responses, and cause disease.

The progression of disease during amebiasis is a complex study of host/parasite interactions. Because mucins are the first line of defense against *E. histolytica* invasion, we were specifically interested in understanding how mucins protect the intestinal epithelium from *E. histolytica* and how mucin secretion is regulated during infection. This in turn may further our knowledge of how *E. histolytica* overcomes the protective mucous layer and adheres to the colonic epithelium. In manuscript I, we showed that *E. histolytica* produces the lipid mediator PGE_2. Based on the known cellular responses to PGE_2 in mammals, several lines of evidence suggested that the parasite produces this product: a) amebic secretory components induce mucin secretion and IL-8 production (3, 4) and; b) there is marked suppression of immune responses during invasive disease. To facilitate the study of *E. histolytica*-mucin interactions, we developed an *in vitro* model using the mucin

producing adenocarcinoma cell line LS174T, which has been extensively characterized. As described in manuscript II, mucins isolated from these cells inhibited amebic adherence to target cells and protected LS174T cell monolayers from destruction by *E. histolytica*. In manuscript III, we described for the first time the receptor-coupled signaling involved in PGE_2-mediated mucin exocytosis. Parasite-derived PGE_2 would interact at the EP_4 receptor to stimulate $[cAMP]_i$-dependent mucin exocytosis.

The progression to amebic colitis is preceded by punctate epithelial lesions, invasion, and a generalized inflammatory response. Prior to the development of focal erosions, a localized concentration of trophozoites was observed in the mucus and a concomitant exhaustion of goblet cell mucins occurred (5, 6). Adherence to the mucous layer by *E. histolytica* is dependent on the interaction of the Gal lectin with mucin O-linked sugars: mucins provide the necessary foothold for successful colonization (7, 8). Local exposure of the underlying epithelium to parasite-derived secretagogues (i.e. PGE_2) may deplete mucin stores and render the mucus less protective. The exposed epithelial cells are then "at the mercy" of the adherent trophozoites, which kill cells indiscriminately. Thus, a generalized inflammatory response with progression to amebic colitis occurs only after amebae penetrate pinpoint areas.

The effect of chronic exposure of goblet cells to mucin secretagogues, specifically PGE_2, is unknown. Transient exposure of the epithelium to parasite-derived PGE_2 may be enough to render conditions optimal for invasion. Another mechanism whereby production of PGE_2 by *E. histolytica* may facilitate invasion is by stimulating IL-8 production (9). IL-8 is a chemoattractant and activating factor for neutrophils and infiltration of the lamina propria and mucosal layer by neutrophils is a characteristic of intestinal amebiasis (5, 6, 10). The release of reactive oxygen intermediates and proteolytic enzymes may initiate

tissue injury and contribute to lytic lesions observed in intestinal infections.

During invasive disease, production of PGE_2 by *E. histolytica* may be vital to its survival. The inability of neutrophils, macrophages and T cells to eliminate trophozoites is a hallmark of hepatic amebiasis. Several lines of evidence implicate PGE_2 in the downregulation of immune functions during hepatic disease: inhibition of macrophage production of IL-1 and IFN-γ, inhibition of macrophage expression of Ia molecule which was reversible with indomethacin and suppression of the Th1 cytokine IL-2 during acute disease (11-14). Although the major source of PGE_2 during amebic abscess development is host-derived (from the induction of COX-2), parasite-derived PGE_2 may also contribute to the suppression (15). In agreement, treatment of hamsters with indomethacin decreased abscess size by 30% in hamsters with experimentally induced amebic liver abscesses (16). Interestingly, we showed that amebic COX activity is not affected by indomethacin (Manuscript I) and therefore parasite-derived PGE_2 may have contributed to maintenance of abscess integrity in the hamster liver once host PGE_2 production was suppressed.

The study of mucin biology was facilitated by the derivation of intestinal cell lines. The human adenocarcinoma cell line LS174T expresses *MUC2* mRNA and secretes predominantly MUC2 (17, 18). LS174T cell mucins are biochemically similar to native colonic MUC2 and are present predominantly in an insoluble pellet by guanidinium chloride extraction (19). LS174T cells respond to numerous secretagogues (see Table 1 in Chapter 2) much like colonic goblet cells. Based on these criteria, we used these cells in our *in vitro* model to study *E. histolytica*-mucin interactions. Previous studies described the interaction between the intestinal epithelial cell line Caco-2 and *E. histolyitca* (20, 21). Unfortunately, these studies provided little insight into the protective function of secreted mucins. Mucins isolated from LS174T cells inhibited amebic adherence to CHO cells

similar to rat and human colonic mucins (manuscript II and reference 6). The presence of an intact mucous layer prevented the killing of LS174T monolayers by *E. histolytica* whereas CHO cells, a non-mucin producing cell line, were rapidly destroyed. Antisense constructs of *MUC2* mRNA were recently used to impair MUC2 production in LS LiM6 cells, a derivative of the LS174T cell line (22). Application of this technology could provide a novel approach to demonstrate the protective function of MUC2 mucins in our model system.

A resounding question in the study of prostaglandins in inflammation is, "is increased prostaglandin production good or bad?" Because NSAIDs reduce the proinflammatory symptoms (pain, swelling and fever) associated with increased systemic prostaglandin production, increased production is considered deleterious. However, abrogation of gastrointestinal prostaglandin synthesis may cause gastric or duodenal tissue injury. Moreover, studies addressing the functional roles of prostaglandins and EP receptors in gastrointestinal tissues demonstrated their importance in cytoprotection (23-25). In COX deficient mice, increased colonic injury occurred in response to dextran sodium sulfate-induced colitis, indicating that prostaglandins are important in protecting the epithelium (26). In our study, we described the receptor signaling pathway involved in PGE_2-mediated mucin exocytosis and demonstrated that the EP_4 receptor was linked to mucin exocytosis via $[cAMP]_i$ (Manuscript III). Thus, we have characterized another cytoprotective mechanism of PGE_2. Taken together, the answer to the above question may not be so simple. Instead, the transiency of increased prostaglandin production may the key to understanding its switch from maintaining homeostatic processes to causing pathophysiologic responses. During intestinal inflammation, transient expression of COX-2 with increased PGE_2 production may contribute to tissue repair through cytoprotective mechanisms. As inflammation subsides, so does the increased production of PGE_2. In

contrast, "constitutive" expression of COX-2 expression was observed in colon cancer tissues and inhibition of prostaglandin production with NSAIDs or ablation of the COX-2 gene significantly decreased the number and size of polyps in mice and humans (27-30). The contribution of dysregulated COX-2 to tumorigenesis may include reduced apoptosis of colon cancer cells and increased tumor angiogenesis (31, 32).

The increased PGE_2 output that occurs during intestinal inflammation modulates a variety of intestinal functions involved in protecting the epithelium: 1) decreasing intestinal permeability, 2) stimulating Cl^- secretion and 3) evoking mucin secretion. In agreement, inhibition of prostaglandin synthesis with NSAIDs can exacerbate inflammatory bowel disease symptoms (33). With respect to intestinal amebiasis, secretion of mucins in response to parasite production of PGE_2 may initially protect the colonic epithelium from amebic adherence. However, prolonged exposure to amebic secretory components may deplete stored mucins and perturb ion balances and cause water secretion (34, 35). The resulting secretory response may expose the colonic epithelium to residual pathogens. In agreement, in humans and animal models of infection, amebae invaded in areas where mucus was depleted (5, 6).

In conclusion, we identified a parasite product that could play a key role host/parasite interactions. This study has paved the way to identify the gene that encodes the amebic COX-like enzyme. Identification of the gene would provide novel insight on a potential drug target and further our understanding of the evolution of COX throughout history. We demonstrated experimentally, the importance of an intact mucous layer in protecting colonic epithelial cells from lysis by *E. histolytica* and described the receptor-coupled mechanism whereby PGE_2 evokes mucin secretion. This is crucial to our understanding of how *E. histolytica* modulates intestinal functions during infection. At present, commercially available EP_4 antagonists are under development and would be

useful to future studies in this area. It is now clear that *E. histolytica* exploits several host responses that contribute to the pathophysiology of intestinal amebiasis and facilitate invasion (Figure 1).

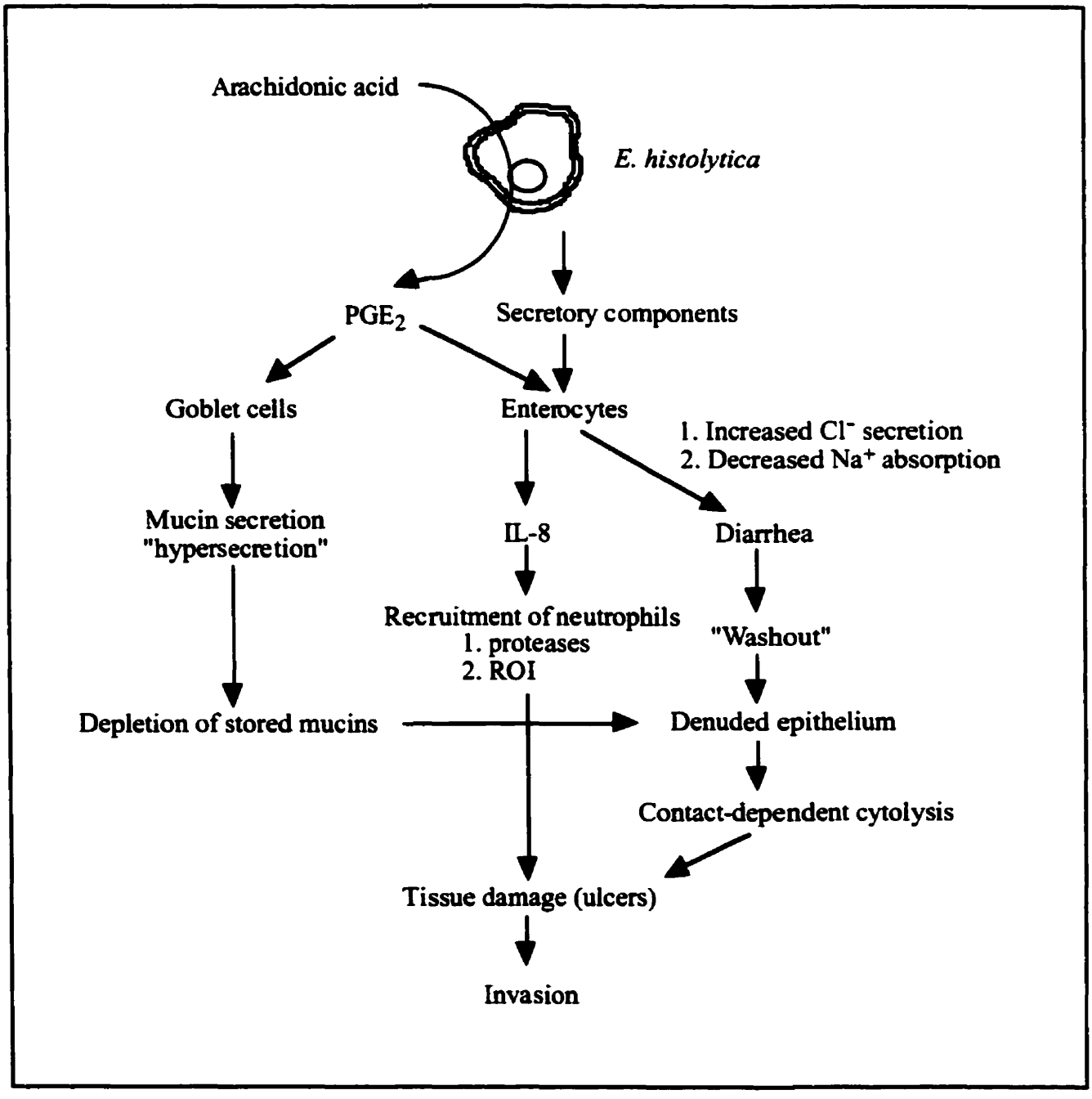

Figure 1. Potential mechanisms used by *E. histolytica* to cause disease

References

1. Hecht G. Innate mechanisms of epithelial host defense: spotlight on intestine. Am J Physiol 1999;277:C351-C358.

2. Forstner JF, Forstner GG. Gastrointestinal mucus. In Johnson LR, ed. Physiology of the gastrointestinal tract. Volume 2, 3rd ed. New York: Raven, 1995:1255-1283.

3. Chadee K, Keller K, Forstner J, Innes DJ, Ravdin JI. Mucin and nonmucin secretagogue activity of *Entamoeba histolytica* and cholera toxin in rat colon. Gastroenterology 1991;100:986-997.

4. Yu Y, Chadee K. *Entamoeba histolytica* stimulates interleukin-8 from human colonic epithelial cell without parasite-enterocyte contact. Gastroenterology 1997;112:1536-1547.

5. Prathap K, Gilman R. The histopathology of acute intestinal amebiasis. A rectal biopsy study. Am J Pathol 1970;60:229-246.

6. Chadee K, Meerovitch E. *Entamoeba histolytica*: early progressive pathology in the cecum of the gerbil (*Meriones unguiculatus*). Am J Trop Med Hyg 1985;34:283-291.

7. Petri Jr. WA, Smith RD, Schlesinger PH, Murphy CF, Ravdin JI. Isolation of the galactose-binding lectin that mediates in vitro adherence of *Entamoeba histolytica*. J Clin Invest 1987;80:1238-1244.

8. Chadee K, Petri Jr. WA, Innes DJ, Ravdin JI. Rat and human colonic mucins bind to

and inhibit adherence lectin of *Entamoeba histolytica.* J Clin Invest 1987;80:1245-1254.

9. Yu Y, Chadee K. Prostaglandin E_2 stimulates interleukin-8 gene expression in human colonic epithelial cells by a posttranscriptional mechanism. J Immunol 1998;161:3746-3752.

10. Baggiolini M, Dewbald B, Moser B. Interleukin-8 and related chemotactic cytokines--CXC and CC chemokines. Adv Immunol 1994;55:97-197.

11. Denis M, Chadee K. *In vitro* and *in vivo* studies of macrophage functions in amebiasis. Infect Immun 1988;56:3126-3131.

12. Salata RA, Martinez-Palomo A, Canales L, Murray HW, Trevino N, Ravdin JI. Supression of T-lymphocyte responses to *Entamoeba histolytica* antigen by immune sera. Infect Immun 1990;58:3941-3946.

13. Wang W, Chadee K. *Entamoeba histolytica* suppresses gamma interferon-induced macrophage class II major histocompatibility complex Ia molecule and I-Aß mRNA expression by a prostaglandin E_2-dependent mechanism. Infect Immun 1995;63:1089-1094.

14. Campbell D, Chadee K. Interleukin (IL)-2, IL-4, and tumor necrosis factor-α responses during *Entamoeba histolytica* liver abscess development in gerbils. J Inf Dis 1997;175:1176-1183.

15. Wang W, Chadee K. *Entamoeba histolytica* alters arachidonic acid metabolism in macrophages in vitro and in vivo. Immunology 1992;76:242-250.

16. Sanchez-Ramirez B, Escalante B, Rosales-Encina JL, Talamas-Rohana P. Role of prostaglandin E_2 in amebic liver abscess formation in hamsters. Prostaglandins 1997;53:411-421.

17. Hollingsworth MA, Strawhecker JM, Caffrey TC, Mack DR. Expression of MUC1, MUC2, MUC3 and MUC4 mucin mRNAs in human pancreatic and intestinal tumor cell lines. Int J Cancer 1994;57:198-203.

18. McGuckin MA, Devine PL, Ward BG. Early steps in the biosynthesis of MUC2 epithelial mucin in colon cancer cells. Biochem Cell Biol 1996;74:87-93.

19. Axelsson MAB, Asker N, Hansson GC. O-glycosylated MUC2 monomer and dimer from LS174T cells are water-soluble, whereas larger MUC2 species formed early during biosynthesis are insoluble and contain nonreducible intermolecular bonds. J Biol Chem 1998;273:18864-18870.

20. Rigothier MC, Coconnier MH, Servin AL, Gayral P. A new in vitro model of *Entamoeba histolytica* adhesion, using the human colon carcinoma cell line Caco-2: scanning electron microscopic study. Infect Immun 1991;59:4142-4146.

21. Li E, Stenson WF, Kunz-Jenkins C, Swanson PE, Duncan R, Stanley SL. *Entamoeba histolytica* interactions with polarized human intestinal Caco-2 epithelial cells. Infect Immun

1994;62:5112-5119.

22. Sternberg LR, Byrd JC, Yunker CK, Dudas S, Hoon VK, Bresalier RS. Liver colonization by human colon cancer cells is reduced by antisense inhibition of MUC2 mucin synthesis. Gastroenterology 1999;116:363-371.

23. Takeuchi K, Yagi K, Kato S, Ukawa H. Roles of prostaglandin E-receptor subtypes in gastric and duodenal bicarbonate secretion in rats. Gastroenterology 1997;113:1553-1559.

24. Sakai H, Kumano E, Ikari A, Takeguchi N. A gastric housekeeping Cl^- channel activated via prostaglandin EP_3 receptor-mediated Ca^{2+}/Nitric oxide/cGMP pathway. J Biol Chem 1995;270:18781-18785.

25. Blikslager AT, Roberts MC, Rhoads JM, Argenzio RA. Prostaglandins I_2 and E_2 have a synergistic role in rescuing epithelial barrier function in porcine ileum. J Clin Invest 1997;100:1928-1933.

26. Morteau O, Morham SG, Sellon R, Dieleman LA, Langenbach R, Smithies O, Sartor RB. Impaired mucosal defense to acute colonic injury in mice lacking cyclooxygenase-1 or cyclooxygenase-2. J Clin Invest 2000;105:469-478.

27. Kargman SL, O'Neill GP, Vickers PJ, Evans JF, Mancini JA, Jothy S. Expression of prostaglandin G/H synthase-1 and -2 protein in human colon cancer. Cancer Res 1995;55:2556-2559.

28. Kutchera W, Jones DA, Matsunami N, Groden J, McIntyre TM, Zimmerman GA, White RL, Prescott SM. Prostaglandin H synthase 2 is expressed abnormally in human colon cancer. Proc Natl Acad Sci USA 1996;93:4816-4820.

29. Giardello FM, Hamilton SR, Krush AJ, Piantadosi S, Hylind LM, Celano P, Booker SV, Robinson CR, Offerhaus GJA. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. New Engl J Med 1993;328:1313-1316.

30. Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hanconck B, Kwong E, Trzaskos JM, Evans JF, Taketo MM. Suppression of intestinal polyposis in $APC^{\Delta 716}$ knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell 1996;87:803-809.

31. Tsujii M, DuBois RN. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. Cell 1995;83:493-501.

32. Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hri M, Dubois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell 1998;93:705–716.

33. Bjarnason I, Hayllar J, MacPherson AJ, Russell AS. Side effects of nonsteroidal anti-inflammatory drugs on the small and large intestine in humans. Gastroenterology 1993;104:1832-1847.

34. McGowan K, Piver G, Stoff JS, Donowitz M. Role of prostaglandins and calcium in the effects of *Entamoeba histolytica* on colonic electrolyte transport. Gastroenterology 1990;98:873-880.

35. Shibayama M, Navarro-Garcia F, Lopez-Revilla R, Martinez-Palomo A, Tsutsumi V. In vivo and in vitro experimental intestinal amebiasis in Mongolian gerbils (*Meriones unguiculatus*) Parasitol Res 1997;83:170-176.