THE UNIVERSITY OF CALGARY

Factors that Modulate Gastric Ulcer Healing

By

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Numerous factors influence gastric ulcer healing. The broad objective of the research described in this dissertation was to gain insight into the effects of new nonsteroidal anti-inflammatory drugs (NSAIDs), bacterial colonization and epidermal growth factor (EGF) on gastric ulcer healing.

Studies were performed to determine the effects of chronic administration of a nitric oxide (NO)-releasing NSAID (NO-NSAID) to rats with pre-existing gastric ulcers. Accelerated gastric ulcer healing was observed following treatment with nitrofenac, an NO-releasing derivative of diclofenac, despite the fact that it suppressed cyclooxygenase activity. Further, the accelerated ulcer healing appeared to be due to NO release, since administration of a nitric oxide donor also markedly accelerated ulcer healing.

Experiments were undertaken to examine the effect of treatment with the selective COX-2 inhibitor, celecoxib, on ulcer healing. Celecoxib exhibited anti-inflammatory activity in the carrageenan-airpouch model in the rat, reducing both leukocyte infiltration and prostaglandin E$_2$ production. However, chronic administration of celecoxib to rats with gastric ulcers markedly impaired ulcer healing.

In the presence of a gastric ulcer in the rat, several different species of bacteria were found to colonize the ulcer site, resulting in delayed ulcer healing. Studies were conducted to characterize the time-course of colonization. Further, specific manipulation of the bacterial species colonizing the ulcers, through the administration of antibiotics or probiotics, influenced healing rates. Previous work had demonstrated that epidermal growth factor (EGF) could prevent bacterial colonization in the rabbit intestine. Taking into consideration the significant levels of bacteria colonizing a gastric ulcer site, EGF was administered to rats with ulcers to determine its ability to influence bacterial levels in the setting of pre-existing...
colonization. Chronic treatment with EGF markedly accelerated gastric ulcer healing. In association with this accelerated healing, a marked decrease in bacterial colonization of the ulcer site was observed.

Overall, these studies highlight the influence of NO, COX-2 inhibition and bacterial colonization on experimental gastric ulcer healing and provide clues to future development of agents that might be used clinically to treat ulcer disease.
I would like to express my sincere gratitude to Dr. John Wallace for providing me with such a unique and rewarding experience. Your creativity, enthusiasm and entrepreneurial approach to science was enlightening and an inspiration.

To the members of Dr. Wallace's Lab, past and present, many thanks for your help and good humour. I leave the lab with many fond memories and friendships.

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<th>Full Form</th>
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<tr>
<td>~</td>
<td>approximately</td>
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<td>©</td>
<td>copyright</td>
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<tr>
<td>μEq.</td>
<td>micro-equivalents</td>
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<tr>
<td>v/v</td>
<td>volume per volume</td>
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<tr>
<td>w/v</td>
<td>weight per volume</td>
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<tr>
<td>°C</td>
<td>degree(s) Celsius</td>
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<tr>
<td>5-LO</td>
<td>5-lipoxygenase</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>CFU</td>
<td>colony forming units</td>
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<td>COX</td>
<td>cyclooxygenase</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>cDNA</td>
<td>complimentary DNA</td>
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<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>bFGF</td>
<td>basic FGF</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>GTN</td>
<td>glycercyl trinitrate</td>
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<tr>
<td><em>H. pylori</em></td>
<td><em>Helicobacter pylori</em></td>
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<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
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<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>ID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>dose required for 50% inhibition</td>
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<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
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All measurements are expressed using Système International units and prefixes.
I am a part of all I have met.

-Lord Tennyson
CHAPTER 1

GENERAL INTRODUCTION

Peptic ulcer is a breach in the gastric or duodenal mucosa associated with acute and chronic inflammation (1). By definition, an ulcer penetrates the muscularis mucosae. The term “peptic ulcer” was used to indicate that pepsin and hydrochloric acid were regarded as the agents that cause the lesions or prevent them from healing (2). “The experience of many gastroenterologists that both gastric and duodenal ulcers tend to heal under types of medical management devised to neutralize the hydrochloric acid of the gastric content during the entire twenty-four-hour period must be regarded as definite evidence that this view is correct” (2). For many years, this dictum of “no acid, no ulcer” dominated thinking on the pathogenesis of peptic ulcer. In 1971, Sir John Vane proposed inhibition of prostaglandin synthesis as the mechanism of action of NSAIDs (3). This discovery, coupled with the observation that as a side-effect, all aspirin-like drugs induced gastrointestinal injury, expanded the scope of peptic ulcer etiology. Just over one decade later, the discovery of the association between colonization of the stomach by Helicobacter pylori and peptic ulcer disease provided yet another advance in understanding the pathogenesis of peptic ulcer disease.

This chapter will serve to review some of the background information that directed the studies described in this dissertation. This will not be a comprehensive review; rather, it will highlight some of the existing knowledge regarding ulcer healing and the role of various modulatory factors. This chapter will conclude with a statement of the objectives that guided the work presented in this dissertation. Additional introductory information will be provided in the chapters that follow.

The gastric mucosa is frequently exposed to noxious stimuli (i.e. acid, proteolytic enzymes, ingested particles and microbes). The maintenance of the integrity of the mucosa is a dynamic equilibrium between the aggressive and
defensive factors. "Mucosal defense" refers to the factors that permit the mucosa to withstand frequent exposure to substances with a wide range of pH, osmolarity, temperature, bacterial products capable of causing inflammatory reactions and agents with detergent properties (4). An imbalance, such that the defensive factors can not counteract the aggressive factors, may result in ulceration.

A gastric mucosal erosion is a defect in the gastric mucosa that may extend to, but does not penetrate the muscularis mucosae. In contrast, an ulcer is a site of injury penetrating through the muscularis mucosae. Acute ulceration often occurs in instances where the ability to maintain normal mucosal integrity is impaired. If the noxious stimulus is removed, or disappears, restorative changes and healing will occur. This is the concept behind the "no acid, no ulcer" therapy. In the continued presence of impaired mucosal integrity and/or a noxious stimulus, ulceration may become chronic. The chronic ulcer often appears as a round defect surrounded by a thickened mucosa covered margin. The base of the ulcer is normally excavated, filled with fibrin, debris and granulation tissue. In many endoscopic studies, ulcers have been defined as a lesion with a diameter >3 mm, without requirements on depth (5). However, much larger ulcers are often observed and most that bleed significantly are between 8 and 30 mm in diameter (5).

Analyses of ulcer healing have been performed in multiple models at multiple sites, including skin ulcers, gastric and intestinal ulcers. Remarkably, regardless of the cause, site or species studied, the mechanisms of ulcer healing show striking similarities (6). Healing is accomplished by filling of the mucosal defect with cells migrating from the ulcer margin, the formation of granulation tissue in the ulcer base and the remodelling of the basement membrane. Factors that accelerate gastric ulcer healing (e.g., growth factors, nitric oxide, prostaglandins) do so by promoting the mucosal defensive mechanisms, such as stimulating mucus secretion and maintaining blood flow, or by decreasing the aggressive factors, such as gastric acid. Many of these modulatory factors are constitutively present and help maintain normal homeostasis within the GI tract. However,
alterations in the levels of these factors may influence the process of gastric ulceration and ulcer healing. Some of these factors will be examined in more detail in the sections that follow.

1.1 Nonsteroidal anti-inflammatory drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely prescribed class of drugs, with annual consumption by more than 30 million people in the United States alone (7). NSAIDs are used for their anti-inflammatory, antipyretic, anti-thrombotic and analgesic properties. NSAIDs inhibit cyclooxygenase activity and therefore the subsequent production of prostanoids (3).

Cyclooxygenase. Eicosanoids are 20 carbon fatty acids that are produced from arachidonic acid via the enzyme cyclooxygenase. Eicosanoid production is initiated by phospholipase A2, which liberates arachidonic acid from membrane phospholipids. The eicosanoids can be divided into two major groups: the leukotrienes and the prostanoids. Leukotrienes are formed by initial conversion of arachidonic acid to leukotriene (LT) A4 by the enzyme 5-lipoxygenase (5-LO), in a calcium- and ATP-dependent reaction (8). LTA4 is then converted into either LTB4 by an enzymatic reaction mediated by LTA4 hydrolase or conjugated with glutathione by LTC4 synthase to form the peptidoleukotrienes (LTC4, LTD4, and LTE4). As the name implies, this latter group contains amino acid moieties (8).

The prostanoids are comprised of the prostaglandins (PG; D, E, F and I series) and thromboxanes (TX). Prostanoids are formed by the initial conversion of arachidonic acid to a common intermediate, PGH2, by the enzyme prostaglandin synthase (PGS), which is also called cyclooxygenase (COX). Prostaglandin synthase performs two independent but sequential reactions. The cyclooxygenase activity converts arachidonic acid to prostaglandin G2 (PGG2). The hydroperoxidase activity then reduces this unstable intermediate to PGH2, the common precursor for all the prostanoids (9). The subsequent synthesis of the
prostanoids is determined by the presence, or lack thereof, of the enzyme that converts PGH₂ to these compounds (e.g., PGI synthase, thromboxane synthase).

The eicosanoids (leukotrienes, prostaglandins and thromboxanes) are shortlived in the bloodstream, with half-lives of seconds to minutes. They act in an autocrine or paracrine manner (10). Cellular recognition and effects of the eicosanoid mediators are attributable to homologous subsets of G-protein associated receptors with seven transmembrane domains (9). The many members of this subfamily have distinguishing patterns of distribution, structural features, pathways of signal transduction and mechanisms for regulation of expression that result in a high level of specificity (9).

NSAID use is associated with serious untoward effects on the gastrointestinal tract. Bleeding and ulceration induced in the stomach by these agents, referred to as “NSAID-gastropathy”, are the most common adverse effects, with an overall prevalence of ulceration in about 25% of patients on long-term NSAID therapy (5). Evidence exists to suggest that NSAIDs induce gastric damage by at least two separate mechanisms: suppression of prostaglandin synthesis and topical irritant effects (11-14). However, topical irritant properties of NSAIDs most likely do not contribute significantly to clinically significant ulceration as NSAID administration by alternate routes (i.e., parenteral or rectal) still results in ulcer formation and bleeding (15,16). Likewise, parenteral administration of NSAIDs to laboratory animals has been shown to cause gastric erosions and ulcer formation (17). Thus, the suppression of prostaglandin synthesis, through inhibition of cyclooxygenase, is a key component of the mechanism underlying gastric ulceration caused by NSAIDs (3).

**Prostaglandins.** The striking ability of prostaglandins to increase the resistance of the gastric mucosa to damage was first noted by Robert in 1976 (18). This discovery lead to extensive research on how the mucosa is able to defend itself from the repeated exposure to a harsh acidic environment and noxious agents. Prostaglandins are pleiotropic in their actions. Prostaglandins can stimulate mucus and bicarbonate secretion. The mucus gel and pH gradient are both important in protecting the gastric mucosal lining from the acid environment of
the stomach lumen. Prostaglandins E and I are potent inhibitors of agonist-stimulated acid secretion in both humans and animals (19,20). Prostaglandins maintain mucosal blood flow by acting as direct vasodilators and can enhance the resistance of the mucosa to injury induced by cytotoxins (21). Prostaglandins are also capable of inhibiting leukocyte recruitment and the subsequent activation and release of inflammatory mediators. In this way, they can suppress the generation of reactive oxygen metabolites that may contribute to tissue injury, and the release of chemotaxins such as IL-8 and LTB₄ (10,22). Prostaglandins are capable of modulating the activity of immunocytes within the mucosa of the gastrointestinal tract. They have been shown to suppress acute mast cell-dependent inflammation (23) as well as inhibiting the release of platelet-activating factor from mast cells (10).

**NSAIDs and ulcer healing.** In non-ulcerated gastroduodenal mucosa, cell proliferation has been shown to be increased with NSAID administration (24). This increase in cell proliferation has been suggested to occur as a consequence of shedding of damaged surface epithelial cells. In contrast, cell proliferation in the ulcer margin is significantly decreased with NSAID administration, subsequently resulting in delayed ulcer healing (25). The ability of NSAIDs to interfere with ulcer healing has been well documented both in humans (25-27), and in models of gastric ulcer in the rat (28-30). There are several mechanisms through which NSAIDs may impair ulcer healing. Prostaglandin inhibition, decreased wound contraction, angiogenesis and blood flow to the ulcer margin have all been implicated as factors (31). A marked increase in glycosaminoglycan synthesis at the ulcer site has been observed in indomethacin-treated rats (31). The significantly elevated synthesis of this component of the extracellular matrix has been suggested to effect the structural organization of the granulation tissue, thereby inhibiting ulcer contraction and ultimately ulcer healing (31). In both humans and experimental models, NSAIDs have been shown to significantly decrease angiogenesis in the ulcer bed (6). NSAIDs can reduce gastric mucosal blood flow (32), and NSAIDs may stimulate neutrophil infiltration into the regenerating mucosa. Indomethacin administration during the healing phase of
acetic acid-induced gastric ulcers in rats significantly impaired healing and a marked neutrophil infiltration into the lamina propria of the regenerating mucosa was observed at the ulcer site (33).

The pernicious effects of NSAIDs present a dilemma in terms of the treatment of patients with arthritis who develop an ulcer. The delaying effect of the NSAID on ulcer healing and inhibition of platelet aggregation (due to inhibition of thromboxane synthesis) may contribute to bleeding from ulcers and this could lead to life-threatening hemorrhage. On the other hand, many patients depend upon the pain relief offered by NSAIDs.

One of the approaches used in an attempt to diminish gastric injury associated with NSAID use was concomitant therapy with a prostaglandin. Initial work looked at the administration of exogenous prostaglandins, such as the prostaglandin E₁ analogue, misoprostol, to prevent or accelerate the healing of NSAID-induced gastric ulcers (34,35). However, other studies suggested that the healing of ulcers by exogenous prostaglandins is impaired if NSAID use is not stopped (36). The greatest limiting factor with misoprostol therapy was the gastrointestinal side effects, particularly diarrhea and abdominal cramping. These symptoms occurred in 20 to 40% of patients treated with the highest approved dose and often lead to cessation of therapy (37).

Taking into consideration the size of the market for NSAIDs (5) and their profound adverse effects, numerous strategies have been employed in recent years to create new anti-inflammatory and analgesic drugs that spare the gastrointestinal tract. The coupling of a nitric oxide-releasing moiety to a standard NSAID (NO-NSAID), and the development of selective inhibitors of cyclooxygenase-2 are two approaches that have been taken.

1.2 Nitric Oxide

Nitric oxide (NO) accounts for the activities originally ascribed to "endothelium-derived relaxing factor", an autocoid that was first described by
Furchgott and Zawadzki (38). NO is produced by virtually all mammalian cells. NO is synthesized from L-arginine via NO synthase (NOS), which exists in three distinct isoforms. The constitutive isoform of NOS (cNOS), is a calcium/calmodulin dependent enzyme expressed in neurons (type I, nNOS) and in endothelial cells (type III, eNOS). Whereas the inducible isoform of NOS (type II, iNOS) is expressed in many cell types including endothelial cells, macrophages and neutrophils following exposure to cytokines and lipopolysaccharide (LPS) (39).

NO is a critical mediator of mucosal defence. Similar to what is observed with prostaglandins, the inhibition of NO synthesis results in exacerbation of injury (40,41). NO is important in regulating epithelial barrier function in the GI tract; inhibition of NO synthesis results in a marked increase in epithelial permeability that can be reversed with NO donors (42-46). NO is able to increase both mucus and electrolyte secretion (47-52). Mucus protects the mucosa from acid, pepsin and abrasion as well as limiting the penetration of luminal epithelial surface. NO is capable of modulating immunocytes within the microvasculature (44,53-55). NO decreases P selectin expression on the endothelium, decreases the adherence and activation of neutrophils and platelets, and stabilizes mast cells resulting in decreased degranulation (44,53-59). NO also has inhibitory actions on gastric acid secretion by suppressing the release of histamine from enterochromaffin-like cells (ECL) (60). Further, NO is a potent vasodilator (39,61-64) and has been demonstrated to inhibit endothelin-1 release from the endothelium (65). Nitric oxide has also been reported to be a free radical scavenger (66-69).

Nitric oxide appears to be capable of modulating the healing of gastric ulcers. Studies reported in 1993 demonstrated that the administration of a nitric oxide donor, glyceryl trinitrate, markedly accelerated gastric ulcer healing in the rat (70). Moreover, the administration of a NO synthesis inhibitor impaired healing. This has been confirmed using a different ulcer model in the rat (71). Ulcer healing is significantly delayed by exposure to cigarette smoke (72) and it has been suggested that the decreases in gastric blood flow and angiogenesis associated with cigarette smoke exposure may be due to reductions in cNOS expression and activity (72). Further evidence for the role of NO in ulcer healing came from Akiba
et al. (73). These authors found that inhibition of iNOS delayed gastric ulcer healing in the rat, and proposed that iNOS inhibition resulted in a marked accumulation of inflammatory cells at the ulcer site and a subsequent increase in oxygen free radicals and cytokines that were deleterious to ulcer healing (73). Healing of anal fissures associated with Crohn’s disease has also been reported to be accelerated by an NO donor (74). NO synthesis in wounds has been suggested to be critical to collagen accumulation and the subsequent acquisition of mechanical strength (75).

**Nitric oxide (NO)-releasing NSAIDs.** The linking of an NO-releasing moiety to a standard NSAID is one approach to reducing the GI toxicity of NSAIDs (76-78). The rationale behind this strategy is that the nitric oxide, by maintaining gastric mucosal blood flow and preventing leukocyte adherence within the gastric microcirculation, may counteract the detrimental effects of cyclooxygenase suppression thereby preventing mucosal injury. The compounds, flurbiprofen nitroxybutylester, ketoprofen nitroxybutylester and diclofenac nitroxybutylester (nitrofenac) have been shown to have comparable anti-inflammatory activity to their parent NSAIDs in models of acute (76,77) and chronic (79) inflammation in the rat. Furthermore, despite inhibiting COX as effectively, these same compounds produced significantly less gastric injury than the parent NSAID with both acute and chronic administration (76-79). Moreover, marked elevations in plasma levels of nitrate/nitrite were observed within an hour of administration of these compounds, (76,77) which is consistent with the release of nitric oxide (76).

Studies examining the ability of these compounds to prevent gastric damage have shown that the administration of naproxen nitroxybutylester or NCX-4016 (an NO-releasing aspirin derivative) provide protection in the stomach against topical irritants (80,81). Given the ability of NO to moderate gastric ulcer healing and the preliminary results demonstrating reduced GI toxicity with these compounds, the potential existed that administration of an NO-NSAID in circumstances of pre-existing ulceration may be beneficial to the healing process.
1.3 Cyclooxygenase-2

In 1972, Flower and Vane (82) demonstrated that cyclooxygenase preparations from the brain were more sensitive to the effects of paracetamol than those from the spleen and suggested the existence of a second isoform of COX. The first pharmacological evidence for a second isoform of COX came from Whittle et al. (83) when they reported differential effects of NSAIDs on COX activity in various tissues. In the late 1980's, a cyclooxygenase activity that could be stimulated by interleukin-1 (IL-1) or endotoxin and suppressed by glucocorticoids, both in vitro and in vivo, was described (84-86). This finding supported the suggestion of a second isoform of the enzyme cyclooxygenase. In 1991, the second isoform of cyclooxygenase was cloned (87-91). COX-2 is 60% homologous to COX-1, and the two isozymes appear to have approximately the same affinity and capacity to convert arachidonic acid to prostaglandins (92,93).

COX-1 is the predominant isoform found in virtually all tissues under basal conditions, including the gastrointestinal tract (94). COX-2 is expressed at low levels in most cells, including the human stomach and intestine (95). Higher levels of expression have been noted in the brain and renal cortex, under basal conditions (93). However, following stimulation with cytokines (i.e., IL-1, tumor necrosis factor α (TNFα)), endotoxin, mitogen (i.e., serum), or growth factors (TGFβ, FGF, PDGF, EGF), COX-2 is strongly induced at sites of inflammation in a variety of cells (93). The constitutive expression of COX-1 in most tissues and the rapid increase in COX-2 levels at sites of inflammation led to the proposal that the COX-2 isoform accounts for the production of pro-inflammatory prostaglandins.

Selective COX-2 inhibitors and ulcer healing. The theory that COX-2-derived prostaglandins were responsible for driving inflammatory responses while COX-1-derived prostaglandins performed "housekeeping" functions led to the suggestion that a selective inhibitor of COX-2 would suppress prostaglandin synthesis at sites of inflammation but would spare constitutive prostaglandin synthesis in other tissues, such as the gastrointestinal tract (85). All the NSAIDs
currently available inhibit both isoforms of COX, although the ratio of inhibitory activities on the two enzymes differ significantly among the compounds and depending on the system used to analyze their effects (96-98). Recent research has focused on the development of compounds that are highly selective for the COX-2 isoform.

Preliminary studies in healthy animals and humans have demonstrated that COX-2 selective inhibitors are indeed anti-inflammatory and exhibit reduced ulcerogenic tendencies (85,99-103). However, evidence exists to suggest that COX-2 may help prevent damage in the stomach, and in situations of pre-existing inflammation, the administration of selective COX-2 inhibitors is deleterious (21,104-108). Davies et al. (109) demonstrated a rapid induction of COX-2 in the rat stomach as early as 1 hour following administration of aspirin or indomethacin. This increase in COX-2 could be prevented by the concomitant administration of a prostaglandin, suggesting that reduced prostaglandin levels subsequent to NSAID administration were responsible for stimulating COX-2 expression (109). Similar increases in COX-2 expression following NSAID administration have been observed with rat mesangial cells and chicken embryo fibroblasts (110,111). This rapid induction may serve to protect the mucosa from damage. Gretzer et al. (112) examined the effect of administration of several selective COX-2 inhibitors (NS-398, DFU, 745,337), on the adaptive cytoprotection induced by the mild irritant, 20% ethanol in the rat stomach. Administration of these drugs at anti-inflammatory doses prevented the protection conferred by a mild irritant to subsequent exposure to a noxious substance (112).

COX-2 appears to be important in circumstances of pre-existing inflammation. Experimental colitis in the rat has been shown to be accompanied by a marked upregulation of COX-2 mRNA and protein (21). In parallel with increased levels of the COX-2 enzyme, prostaglandin synthesis was approximately 25-times higher than that observed in the normal colon, and the vast majority of this increase in prostaglandin synthesis was attributable to the up-regulation of COX-2 (21). Administration of selective COX-2 inhibitors (etodolac, nabumetone, L745,337) resulted in a significant inhibition of mucosal prostaglandin synthesis
COX-2 also appears to be critical in promoting ulcer healing. Significant induction of COX-2 mRNA and protein have been demonstrated in gastric erosions and ulcers in the mouse stomach (104). Concomitant increases in prostaglandin synthesis have also been noted and ascribed to the presence of COX-2 (104). Administration of NS-398, a selective inhibitor of COX-2 significantly impaired ulcer healing in the mouse model (104). Similar results have been reported using NS-398 in the rat (105,113). Schmassmann et al. (107) extended these findings with the report that L745,337, another selective COX-2 inhibitor, also markedly delayed ulcer healing using a different rat model. Notably, this inhibition of ulcer healing was observed with doses of L745,337 previously shown to be selective for COX-2 (107). Nimesulide, yet another compound reported to be COX-2 selective, has been shown to significantly augment the severity of stress-induced gastric ulcers in the rat (108). Thus, preliminary data suggest that COX-2 is critical in reducing inflammation. The effect of COX-2 inhibitor administration on healing of gastric ulcers will be addressed in Chapters 4 and 7.

1.4 Bacterial Colonization and Ulcer Disease

The discovery of the association between colonization of the stomach by Helicobacter pylori and peptic ulcer disease has provided an impetus for research into this area. Helicobacter pylori infection of the gastric mucosa results in delayed ulcer healing and is the main cause of ulcer relapse (114). This causal role for H. pylori is clearly indicated by the fact that eradication of the bacterium dramatically reduces ulcer recurrence (114). Despite intensive work in the area of H. pylori-induced gastric inflammation for over a decade, the mechanisms through which H. pylori induces ulceration within the GI tract and/or interferes with ulcer healing are not fully understood. Multiple factors appear to play a role, but a definitive mechanism has not been identified. However, the possibility exists that bacteria other than those of the Helicobacter species could contribute to gastric ulceration if
they are capable of colonizing the stomach, or an ulcer site, and this colonization may influence the natural history of an ulcer.

Bacterial colonization of gastric ulcers was demonstrated as long ago as 1874 (115). Bacterial isolates were found in peptic ulcers of patients with both normal and decreased acid secretion. Shortly thereafter, it was reported that oral or parenteral administration of *Staphylococcus pyogenes* to guinea pigs resulted in gastric ulcers (116). This could be reproduced with several different species of bacteria (116). Rosenow (117-119) pioneered work examining the role of streptococci in ulceration. He was able to isolate streptococci from patients with peptic ulcers. This was confirmed by Saunders in 1930, with isolation of α-streptococcus from human ulcers (120). Around the turn of the century, the presence of spiral organisms in gastric glands and parietal cells of dog stomach were recorded (121). However, up until the successful culturing of the spiral bacterium, *Helicobacter pylori*, in 1982 (122) little attention had been paid to descriptions of bacterial organisms in biopsy specimens of human gastric mucosa (123). It is now recognized that there is a direct association between *H. pylori* colonization and peptic ulcer disease, and perhaps gastric cancer (124).

How is it that bacteria are able to colonize the stomach? *H. pylori* is specially adapted to survive the acidic environment of the stomach, and evidence suggests that some strains carry virulence factors that promote ulceration (125,126). However, preexisting injury to the gastric mucosa may be a permissive factor for *H. pylori* colonization, just as it is for *Streptococcus* or *Candida* (27,115,117,120,127,128). For example, *H. pylori* is not capable of colonizing the normal rat stomach but, if given to rats twice-daily for a week after an ulcer had been induced, heavy colonization could be observed (129). Li et al. (130) extended these findings by demonstrating a similar delay of ulcer healing in rats inoculated with a vacA⁻ and cagA⁻ strain of *H. pylori*. In experimental models, bacteria appear to be important in exacerbating mucosal injury induced in the stomach or small intestine by NSAIDs (76,131,132). The presence of endotoxin may influence the ability of bacteria to impair ulcer healing. Endotoxin (bacterial
lipopolysaccharide; LPS) is a component of the outer membrane of gram-negative bacteria. Colonization with gram-negative bacteria may contribute to the chronicity of gastric ulcers. In acetic acid-induced ulcers in the rat, daily administration of E. coli LPS has been demonstrated to result in a dose-dependent delay in ulcer healing (133). The impaired ulcer healing was associated with decreased gastric blood flow, increased expression of pro-inflammatory cytokines and an imbalance in the ratio between the pro- and anti-apoptotic peptides, favouring apoptosis (133). LPS can stimulate the production of TNFα from mononuclear phagocytes (134,135). TNFα is capable of stimulating the production of other cytokines such as IL-1, IL-2, IL-6 and IL-8 (135). TNFα can amplify tissue injury through the ability to regulate expression for other inflammatory mediators including LTB₄ and PAF (10). Thus, colonization with gram-negative bacteria may amplify tissue injury.

Are all bacteria bad? In 1977, Dwayne Savage examined the gastrointestinal tract of rodents and noted that the gastric mucosa was heavily colonized by the acid-resistant Lactobacillus species. Lactobacilli are also the predominant bacteria found in the human fasting stomach, though in small amounts (0 to 10³ per mL of fluid) (136). Lactobacillus spp. have been shown to be beneficial in the gastrointestinal tract. Kabir et al. (137) found that Helicobacter pylori could not colonize Lactobacillus salivarius infected gnotobiotic BALB/c mice, whereas germ free mice were heavily colonized with H. pylori with the subsequent production of active gastritis. In addition, the administration of L salivarius after H. pylori inoculation was able to eliminate colonization. This ability of Lactobacillus spp. has led to the suggestion that lactobacilli could find therapeutic use, perhaps against H. pylori (137). Lactobacillus has also been shown to prevent the development of acetic acid-induced colitis in the rat (138). Lactobacilli are capable of preventing the growth of potentially pathogenic bacteria and the mechanism has been attributed to their ability to compete for nutrients, to the production of antimicrobial agents such as bacteriocins and metabolic end-products, and the stimulation of immunity (138,139). Thus, the presence of some species of bacteria in the GI tract may serve a beneficial role.
1.5 Growth Factors

In general, peptides designated as peptide growth factors are distinguished from other peptides, such as cytokines, by the relative importance of their effects on growth when compared to other effects that they may exert. Growth factors often exhibit trophic effects on target tissues in an autocrine, paracrine or juxtacrine manner (140). This proximity of action differentiates the growth factors from the hormones, whose target cells or tissues are a distance from the site of production.

Numerous peptide growth factors are intimately involved in the GI tract with both the maintenance of mucosal integrity and the stimulation of repair following a breach in the mucosa. Peptide growth factors in the gastrointestinal tract may be classified into several distinct families based on structural homologies and disparities (141). These include the epidermal growth factor (EGF) family, the transforming growth factor β (TGFβ) family, the insulin-like growth factor (IGF) family, and the fibroblast growth factor (FGF) family. In addition to these families, a smaller number of peptides have been identified within the gastrointestinal tract that exhibit growth-stimulatory properties yet lack structural homology to other growth factors, such as hepatocyte growth factor (HGF) and platelet-derived growth factor (PDGF) (141).

The epidermal growth factor family. Epidermal growth factor (EGF) was originally isolated from mouse salivary glands as a factor capable of inducing the premature eruption of teeth and opening of eyelids in mice (142). This factor has since been shown to be capable of promoting epithelial cell proliferation in a wide variety of tissue and organ culture systems (143). The EGF family is comprised of at least seven different peptides sharing sequence homology, affinity for the same receptor and a similar spectrum of biological activity, most notably, the mitogenic activity of EGF (141). Peptides expressed in the gastrointestinal tract include EGF, transforming growth factor-α (TGFα), amphiregulin and betacellulin (141). All the EGF-related peptides are synthesized as larger, glycosylated, transmembrane precursor proteins with an extracellular domain that is released by proteolytic
cleavage (141). All the mature EGF-related peptides released from the membrane show 100% conservation of six cysteine residues that contribute to their tertiary structures by the formation of three disulfide bonds (143). The particular spacing of these cysteines and intervening amino acids defines the peptide as a member of the EGF-related family (140). The presence of these three bonds creates a unique structure among the EGF-related peptides that permits their high affinity binding to the EGFR (143).

The EGFR is a 170-kilodalton protein that consists of a cell surface ligand-binding domain, a single hydrophobic transmembrane domain and a highly conserved tyrosine kinase domain (143). Ligand binding results in receptor homodimerization, phosphorylation of key tyrosine residues, activation of intracellular signaling systems and ultimately DNA synthesis and cellular growth (140,144).

**Epidermal growth factor.** EGF is a 53 amino acid polypeptide that is synthesized mainly in the submaxillary glands and Brunner's glands in the duodenum (145). EGF is found in most bodily fluids, including saliva, gastric, duodenal and pancreatic secretions and milk (141). Large amounts of EGF may be found in the lumen of the GI tract (12). EGF exerts a variety of actions on the gastric mucosa and is intimately involved in its repair.

**Mitogenic effects.** The wide distribution of the EGFR and EGF throughout the GI tract is indicative of the potent ability of EGF to stimulate proliferation in a variety of cell and tissue types. Healing of gastroduodenal ulcerations requires epithelial cell proliferation, to provide cells for the re-epithelialization of the lesion and the reconstruction of glandular structures within the ulcer bed. Growth of granulation tissue at the base of the ulcer is required to provide connective tissue and microvessels for reconstruction of the lamina propria and the microvascular network. EGF has been shown to be capable of stimulating proliferation in a variety of non-transformed and transformed gastrointestinal cell lines (146). Enhanced proliferation may be observed in gastric cells, as well as in selected non-epithelial cell types present in the GI tract, such as lamina propria lymphocytes and mesenchymal cells (141). EGF modulates cell proliferation and phenotype by the
activation of signal transduction pathways, such as the Ras/Raf/MAP kinase pathway and the JAK/STAT signaling pathway that ultimately result in the transcription of various genes involved in cell proliferation (144).

**Non-mitogenic effects.** Next to the mitogenic ability of EGF, the acid inhibitory activity of EGF may be the physiological effect that has received the most attention. The clinical observation of the low incidence of peptic ulceration during pregnancy led to the finding that extracts of urine from pregnant women had beneficial effects on the healing of chronic ulcers in Mann-Williamson dogs (147). Subsequently it was reported that not only urine from pregnant women but also that from all females and males contained a potent inhibitor of acid secretion (148). This anti-secretory agent was called urogastrone (149) but has since been demonstrated to be the same peptide as EGF (150). With the exception of one *in vitro* study that showed stimulation (151), all studies have reported that EGF administration can inhibit acid secretion (143). Inhibition of agonist-stimulated acid secretion with EGF has been demonstrated in humans (152), dogs (153) and rats (154) and *in vitro* in parietal cells and/or gastric glands from guinea pigs (155), rat (156) and rabbit (151). *In vitro* results suggest that EGF acts directly on the parietal cell to reduce acid secretion (143). The importance of acid inhibition in ulcer healing may be demonstrated by the wealth of evidence from human studies (157,158) and animal models (29,159) that suppression of acid secretion results in accelerated ulcer healing.

Reduced gastric blood flow can lead to mucosal damage and retard repair (4). EGF is a potent vasodilator in the gastric mucosa (160,161). The ability of EGF to increase gastric blood flow has been demonstrated to be important in minimizing damage (161,162) as well as aiding in repair (162). Blood flow is critical to the delivery of oxygen, nutrients and growth factors to the ulcer as well as limiting further damage by removing noxious substances.

EGF is capable of altering the production and secretion of mucus in the GI tract. EGF increases goblet cell numbers (163) as well as stimulating the synthesis and secretion of mucus in animal (164) and human studies (165). Further, it appears that EGF derived from salivary secretions is important in the maintenance
of normal mucin levels, as sialoadenectomized rats had reduced mucin levels and this could be corrected by EGF administration (166). Increased production and secretion of mucus helps protect the mucosa from aggressive factors such as acid and pepsin.

EGF is capable of activating the gastric mucosal genes encoding for the cyclooxygenases, thereby resulting in increased mucosal generation of prostaglandins (167-169). As discussed in Section 1.1, prostaglandins are critical mediators of mucosal defence in the GI tract.

EGF has been shown to aid in the restoration of absorptive function following injury by stimulating an increase in brush-border surface area (170), up-regulating electrolyte and nutrient transport (171), increasing phospholipid synthesis (172), and the uptake of glucose (173), galactose (174) and glycine (174). Granulation tissue synthesis may also be aided by EGF as it is a potent stimulant of fibroblasts and collagen synthesis (140,175).

**Bacterial infection.** The ability of EGF to modulate mucosal repair via mitogenic and non-mitogenic actions is well recognized. EGF may also play a role in preventing bacterial colonization in the GI tract. Multiple studies (163,176,177) have demonstrated the ability of EGF to prevent bacterial translocation from the gastrointestinal tract to distant organs when intestinal mucosal integrity is compromised. Furthermore, EGF is capable of preventing the colonization of enteropathogenic *E.coli* within the GI tract and the associated diarrhea, disruption of microvilli and reduced activities of intestinal maltase and sucrase (178). While the ability of EGF to aid in the repair of damaged mucosa has long been recognized, the finding that EGF may prevent bacterial colonization and associated disturbances in function suggested a new means through which ulcer healing may be influenced.
1.6 Objectives

Four studies have been compiled to form the body of this dissertation, and these are presented in chapters four through seven. The broad objectives of the work contained herein were:

(1) To determine the effects of a nitric oxide (NO)-releasing nonsteroidal anti-inflammatory drug (NSAID) on the healing of pre-existing gastric ulcers in rats.

(2) To describe and characterize the bacterial colonization that occurs in the presence of a gastric ulcer in the rat, and the effects of manipulation of the bacterial species present.

(3) To study the effects of epidermal growth factor (EGF) administration on bacterial colonization and ulcer healing in the rat.

(4) To examine the anti-inflammatory effects of a dual cyclooxygenase/lipoxygenase inhibitor, a selective COX-2 inhibitor and a 5-lipoxygenase inhibitor and their influence on gastric ulcer healing.
CHAPTER 2

GENERAL MATERIALS AND METHODS

Central to the study of factors that may influence gastric ulcer healing is a simple, reproducible model. In this chapter, the animals used, the method of ulcer induction and ulcer area determination are described in detail. Eicosanoid synthesis, both in serum and gastric tissue, provides an index of the ability of NSAIDs to inhibit the cyclooxygenase enzymes. As the role of NSAIDs on ulcer healing is dealt with in two of the chapters, descriptions of sample collection and a general overview of the ELISAs used are included. Bacterial culturing, in order to quantify bacteria colonizing the gastric ulcer site, was essential to the study examining the impact of bacteria on ulcer healing. The methods employed for culturing and determining levels of colonization are outlined. Further, many of the agents used in this dissertation may have the ability to alter acid secretion. As alterations in acid secretion may have an impact upon ulcer healing, the technique utilized for measuring basal and pentagastrin-stimulated acid secretion is discussed. Additional techniques employed will be addressed in the pertinent chapters.

2.1 Animals

Male, Wistar rats weighing 175-200 grams were obtained from Charles River Laboratories (St. Constant, PQ). Animals were housed in transparent polycarbonate cages at 22°C in a room with a 12-hour light/dark cycle at the Animal Resource Centre, University of Calgary. Animals were allowed free access to standard laboratory chow and drinking water, except where noted. All experimental protocols utilized were approved by the Animal Care Committee at
the University of Calgary and are in accordance with the guidelines of the Canadian Council on Animal Care.

2.2 Gastric ulcers

**Gastric ulcer induction.** The ulcer model that formed the foundation of the studies examining factors that influence gastric ulcer healing was the acetic acid-induced gastric ulcer model. Gastric ulcers were induced in rats using a modified version of the model originally described by Okabe and Pfeiffer (179). Briefly, rats were deprived of food, but not water, for 18-24 hours prior to the gastric ulcer induction procedure. Rats were anesthetized with 5% (v/v) halothane (Halocarbon Laboratories, River Edge, NJ), a midline laparotomy was performed, the stomach was gently exteriorized and held stationary using forceps gripping portions of the forestomach and duodenal omentum. The barrel of a 3-mL syringe, which had been cut and then filed smooth, was placed on the anterior serosal surface of the stomach in the corpus region. 0.5 mL of 80% (v/v) acetic acid was instilled into the syringe barrel so that it made contact with the serosa. After 1 minute, the acetic acid was removed by aspiration, and the region to which it had been applied was washed several times with sterile saline. The area exposed to acetic acid was 60 mm². The stomach was re-internalized and the incision sutured with Ethicon® 3-0 sterile, non-absorbable surgical suture. The rats were then returned to their cages and given food and water *ad libitum*.

The sham-ulcer induction procedure was identical to the ulcer induction procedure described above, except that distilled water, rather than acetic acid, was instilled into the barrel of the syringe and allowed to contact the serosal wall of the stomach.

**Ulcer area determination.** On the day that ulcer area was to be determined, rats were killed either by cervical dislocation or pentobarbital overdose. The stomach was removed, cut along the greater curvature and pinned
out on a wax block. A paper grid with a known area (25 mm²) was placed alongside the ulcer, which was then photographed. Ulcer area was determined by planimetry, using 5x enlargements of the photographs. The area of ulceration in pixels was converted to square millimeters with the paper grid as a reference, using a digitizer palette and Sigma-Scan software (Jandel Scientific, Corte Madra, CA, USA). All planimetric determinations were performed using coded photographs such that the observer was unaware as to what treatment the rats had received.

2.3. Eicosanoid synthesis

**Serum collection.** In platelets, thromboxane A₂ (TXA₂) is generated almost exclusively via the cyclooxygenase (COX)-1 pathway (180). The stable hydration product, TXB₂, can be measured using an enzyme-linked immunosorbent assay (ELISA) to provide an index of COX-1 activity. Under pentobarbital-induced anesthesia (65 mg/kg), ~1.5 mL of blood was collected from the descending aorta. 1 mL of the blood was transferred to a glass culture tube and incubated for 45 minutes at 37°C. Following the incubation, 100 μL of indomethacin (0.1 mg/kg; Sigma Chemical Co., St. Louis, MO) was added to the tube to prevent further thromboxane production and the samples were placed on ice. Serum was separated by centrifugation (2200 rpm, 10 min, 4°C) of the samples. Serum was transferred to Eppendorf tubes and frozen at −20°C until assay.

**Tissue collection.** Tissue samples (100-300 mg wet weight) were excised from the corpus region of the stomach. The tissues were placed in Eppendorf tubes containing 1 mL of 10 mmol/L sodium phosphate buffer (pH 7.4) and finely minced with scissors for 30 seconds to maximally stimulate eicosanoid production. The samples were incubated in a shaking water bath (37°C) for 20 minutes, and then centrifuged at 14,000 rpm for 30 seconds in a microfuge (Eppendorf,
Hamburg, Germany). The supernatants were collected and frozen at -20°C until assayed for prostaglandin E₂ or leukotriene B₄, using an ELISA.

**Eicosanoid ELISAs.** Thromboxane B₂, prostaglandin E₂ and leukotriene B₄ were measured by ELISA. ELISA kits were obtained from Cayman Chemical Company (Ann Arbor, MI), and used according to the manufacturer's instructions. The ELISA kits were based on competitive binding between the eicosanoid in the sample and an eicosanoid-acetylcholinesterase conjugate. Ellman's reagent, which contains the substrate to acetylcholinesterase, was added to the wells and the product of this enzymatic reaction had a distinctive yellow color. Serum samples were diluted 1:50, 1:100, 1:400, and 1:800 and tissue samples were diluted 1:2. Optical density (412 nm) was determined by spectrophotometry. The eicosanoid concentrations in samples were interpolated from the standard curve of absorbance values plotted as a function of known concentrations of the eicosanoids.

### 2.4. Bacterial culturing

**Gastric tissue collection.** Rats were killed by cervical dislocation and a laparotomy was performed using aseptic technique. The stomach was removed, opened along the greater curvature and rinsed with sterile phosphate-buffered saline (PBS; pH 7.4). Tissue samples of approximately 150 mg were obtained from either the ulcer, or a contralateral site in the stomach, as denoted. The tissue samples were placed into sterile tubes containing 5 mL sterile PBS (pH 7.4) and weighed. The weight in grams was recorded. The tissues were homogenized (Polytron homogenizer; Brinkmann Instruments, Rexdale, ON) and serial dilutions of the suspension were made.

**Plating and bacterial number determination.** Serial dilutions of the bacterial suspension (100 μL) were then plated onto MacConkey agar or trypticase soy agar (BBL media; Becton Dickinson Co., Cockeysville, MD) for the culturing of
gram negative aerobic bacteria or total aerobic bacteria, respectively. The MacConkey and trypticase soy agar plates were incubated for 18-24 hours at 37°C under aerobic conditions. Culture plates containing between 20 and 200 colony-forming units (CFU) were analyzed to determine bacterial levels. All bacterial culturing materials were obtained from Becton-Dickinson (Cockeysville, MD).

2.5. Gastric acid secretion

**Gastric perfusate sample collection.** Gastric acid output was measured using a continuous gastric perfusion system, as previously described (181). Rats were fasted (2 per cage) for 24 hours prior to the measurement, with free access to drinking water. Anesthesia was induced with urethane (7.0 mL/kg of a 25% solution prepared in 0.9% sterile saline). The rats remained on homeothermic blankets, to maintain their body temperature at 37°C, throughout the experiment. A jugular vein was cannulated for administration of pentagastrin and a tracheostomy was performed to ensure a patent airway for the duration of the experiment. An orogastric catheter was inserted and secured with two ligatures around the esophagus. A midline laparotomy was performed and a duodeno-gastric cannula inserted and secured with ligatures. The stomach was gently flushed with approximately 20 mL of saline (37°C) to remove any residual matter.

The stomach was perfused with isotonic saline (37°C) at a rate of 3.0 mL/h using a syringe infusion pump (Syringe Infusion Pump 22; Harvard Apparatus, Cambridge, MA). Following a 15-min stabilization period, perfusate was collected for three 30-minute periods. Following the first collection period (basal secretion), a bolus dose of 20 µg/kg of pentagastrin (Sigma Chemical Co., St. Louis, MO; prepared in 0.9% sterile saline) was administered intravenously, followed by a continuous infusion of pentagastrin at 20 µg/kg·h for 60 minutes. Perfusates were collected for each period from the duodeno-gastric cannula into glass tubes.
**Gastric acid output measurement.** The titratable acidity in each gastric perfusate sample was determined by titration to pH 7.0 using an automated titration system (Metrohm automated titrator, Brinkmann Instruments, Rexdale, ON), and is expressed as micro-equivalents (μEq) per 30 minute collection period.
CHAPTER 3

THE ACETIC ACID-INDUCED GASTRIC ULCER MODEL IN THE RAT

In order to study factors that influence gastric ulcer healing, an experimental model must be adopted that is simple and will reliably produce ulcers that exhibit features characteristic of the clinical condition.

**Animal model.** The ulcer model utilized throughout this dissertation is an adaptation of the acetic acid-induced gastric ulcer model originally described by Okabe and Pfeiffer in 1972 (179). Whereas the original model involved the injection of acetic acid into the gastric wall, we chose to apply 80% acetic acid, within the barrel of a 3-mL syringe, to the serosal surface of the gastric corpus, so as to physically limit the area of tissue that the acetic acid contacted. This serosal application of acetic acid produces gastric ulcers that are grossly, histologically and ultrastructurally similar to true gastric ulcers in humans (182).

**Time-course study.** Serosal application of 80% acetic acid reproducibly resulted in a single, deep, ulcerated area of a fixed size. This ability to produce ulcers of a fixed size at a known location, and then observe alterations in ulcer size subsequent to treatment/manipulation was paramount to studying the role of numerous factors involved with gastric ulcer healing.

As early as one minute after application of acetic acid, an opaque area was visible on the serosal surface of the stomach at the point of acid application. By twenty-four hours, the lesions were somewhat irregular in shape and cellular debris was evident on the surface of the lesion. At this time, the area of the ulcers averaged $\sim$100 mm$^2$ (Figure 3.1). Within the first three days following ulcer induction, grossly visible gastric wall thickening was evident in the area where the acetic acid had been applied. There were distinctly elevated ulcer margins and an ulcer crater that was often covered with a whitish exudate. Figure 3.2 shows a representative gastric ulcer, seven days after ulcer induction. Histologically, the ulcers observed at this time point were characterized by a thick layer of granulation tissue with glandular disorganization at the ulcer margins. A thick layer of mucus
and cellular debris were visible in the ulcer bed. These lesions involved the full thickness of the mucosa and penetrated through the muscularis mucosae (Figure 3.3). In some instances, the ulceration extended into the muscularis propria. Perforation was not observed. Marked infiltration of inflammatory cells could be observed within the ulcer bed and levels of inflammatory cells remained elevated until 21 days after ulcer induction. At no point subsequent to the ulcer induction procedure did animals exhibit signs of pain or illness. They ate, drank and gained weight at a constant rate. In parallel, a gradual decrease in gastric ulcer area was observed over the course of 20 days (Figure 3.1).

**Inhibition of acid secretion.** A representative animal model must also be responsive to medication in a manner similar to that of human condition. Considerable evidence exists for the suppression of acid secretion leading to accelerated gastric ulcer healing in humans (183-185). Acid inhibition aids in ulcer healing by reducing levels of acid and thereby protecting connective tissue cells and extracellular matrix in the ulcer base from gastric acid-pepsin damage (159). Oral administration for one week of an histamine type-2 receptor antagonist, ranitidine, to rats with acetic acid-induced gastric ulcers, resulted in significantly reduced gastric ulcer areas compared to vehicle-treated rats (Figure 3.4).

**Mechanism of ulcer formation.** It has been suggested that the mechanism of ulcer formation, through the application of acetic acid, is related to the development of thrombi in the submucosal vessels and collecting venules resulting in ischemic mucosal necrosis and damage due to vascular congestion, free radical formation and nutrient deficiency to the mucosal structures (186). Clinically, this is may be relevant to the chronic gastric ulcers in humans that are caused by ingestion of nonsteroidal anti-inflammatory drugs. Kitahora and Guth (32) first described thrombus formation, cessation of blood flow and subsequent mucosal hemorrhage following aspirin administration, suggesting ischemia as a pathogenetic factor. Wallace et al. (187-189) extended these findings by showing that neutrophil adherence to the vascular endothelium following NSAID administration is a critical event in NSAID-gastropathy. Inhibition of leukocyte adherence (187,188) or the depletion of circulating neutrophils (189) resulted in a
marked reduction in the severity of NSAID-induced gastric lesions. Whether or not neutrophils contribute to NSAID-gastropathy in humans has yet to be determined conclusively. However, a recent clinical study reported that a markedly elevated peripheral white cell count was a significant risk factor for ulcer development and that NSAID-induced gastropathy may be mediated, in part, by neutrophils (190). It has been estimated that greater than 30 million people in the United States alone take prescription NSAIDs regularly (5). Associated with the use of NSAIDs are adverse effects such as ulceration and bleeding. NSAID-induced gastropathy is associated with mucosal vasoconstriction, vascular congestion, and capillary damage. Thus, the underlying mechanisms of ulcer pathogenesis are similar and so this model should be useful for examination of modulatory factors of ulcer healing in man.
Figure 3.1. Spontaneous healing of gastric ulcers in rats over a 3-week period. Ulcers were induced through serosal application of acetic acid. Groups of rats were killed at various times after ulcer induction, and gastric ulcer area measured planimetrically. The ulcers averaged approximately 100 mm² 1 day after ulcer induction and gradually decreased over the course of the next 20 days. N ≥5 at each time point.
Figure 3.2. Representative photo of an acetic acid-induced gastric ulcer seven days after ulcer induction. The ulcer was induced in the corpus region of the stomach through the serosal application of acetic acid. A distinct ulcer crater and elevated ulcer margins are visible. Ulcer area was determined by calibration against the square which is 25 mm$^2$. 
Figure 3.3. Photomicrograph of a rat gastric ulcer. Granulation tissue covers the ulcer base and mucosa at the ulcer margin (seen on the right) forms a proliferative zone that provides cells for re-epithelialization of the mucosal surface and reconstruction of the mucosal glandular structures.
Figure 3.4. Effect of ranitidine administration on gastric ulcer healing in rats. Ranitidine (100 mg/kg) was orally administered twice-daily starting on the seventh day after ulcer induction. Rats receiving ranitidine for one week had significantly smaller gastric ulcer areas than rats receiving vehicle (dH₂O).

**P<0.01 compared to vehicle-treated animals. Each bar represents the mean ± SEM of 5 (vehicle) or 4 (ranitidine) rats.
NITROFENAC, A NITRIC OXIDE-RELEASING NSAID, ACCELERATES EXPERIMENTAL GASTRIC ULCER HEALING

4.1 Introduction

In addition to the ulcerogenic effects exerted by nonsteroidal anti-inflammatory drugs (NSAIDs) in the gastrointestinal tract, the use of these drugs is limited by their ability to interfere with the healing of ulcers. This phenomenon has been well documented both in humans (25,26) and in models of gastric ulcer in the rat (28-30). This effect of NSAIDs presents a dilemma in terms of the treatment of patients with arthritis who develop a gastric or duodenal ulcer, because the delaying effect of the NSAID on ulcer healing and the possibility that this could lead to life-threatening hemorrhage usually require the patient to cease taking the NSAID.

Numerous strategies have been used in recent years to develop anti-inflammatory and analgesic drugs that spare the gastrointestinal tract. Several groups are attempting to develop NSAIDs that only inhibit the inducible isoform of cyclooxygenase (COX) 2, with the belief that these compounds would exert anti-inflammatory activity but spare gastrointestinal prostaglandin synthesis (85,191,192). COX-2 is widely believed to be expressed only at sites of inflammation, whereas COX-1 is constitutively expressed in many tissues, including the gastrointestinal mucosa and the platelet (85,191). The prostaglandins derived from COX-1 are believed to be responsible for many of the physiological effects of these autacoids, including maintenance of gastrointestinal mucosal integrity (85,191). As ulcers usually have surrounding inflammation, it is possible that COX-2 may be expressed and may be producing prostaglandins that contribute to the healing process. Indeed, recent studies have demonstrated that COX-2 is important in promoting the healing of gastric ulcers in mice and rats, as
increased COX-2 mRNA and protein were observed in the stomach when an ulcer was present (104-107,193) and treatment with selective inhibitors of COX-2 resulted in significant inhibition of ulcer healing (104,105,107).

Another strategy for developing gastrointestinal-sparing NSAIDs is the coupling of a nitric oxide-releasing moiety to standard NSAIDs (76,77,194). The rationale behind this strategy is that the NO released from these derivatives will exert beneficial effects on the mucosa by maintaining gastrointestinal blood flow and inhibiting adherence and activation (i.e., release of reactive oxygen metabolites and proteases) of white blood cells within the gastrointestinal microcirculation (76,77). Both a reduction in gastrointestinal blood flow and stimulation of leukocyte adherence and activation have been implicated in the pathogenesis of NSAID gastropathy, at least in experimental models (194). The effects of these compounds in models of pre-existing gastric ulcers have not yet been assessed, although they have been shown to have reduced toxicity vs. standard NSAIDs in a rat model of colitis (78). Because the NO-releasing NSAIDs suppress COX (types 1 and 2) activity as effectively as the parent NSAIDs (76,77,195), and because it is the suppression of prostaglandin synthesis that is thought to account for the delayed ulcer healing effect of NSAIDs (11,27), it is conceivable that these NO-releasing NSAIDs may impair ulcer healing. On the other hand, Konturek et al. (70) recently reported that an NO donor, glyceryl trinitrate, significantly accelerated the healing of experimental gastric ulcers in the rat, whereas inhibitors of NO synthesis impaired gastric ulcer healing.

In the present study, we have tested the effects of daily treatment with an NO-releasing NSAID, nitrofenac (diclofenac 4-nitroxybutylester), in comparison with its parent compound, diclofenac, on healing of experimental gastric ulcers in the rat. The effects of two compounds that have been reported to be more selective for COX-2 than COX-1, nabumetone and L745,337 (96,192), were also assessed in this model, as was a well characterized, standard NO donor (glyceryl trinitrate).
4.2 Materials and Methods

Effect of cyclooxygenase inhibitors. Gastric ulcers were induced in male, Wistar rats weighing 175-200 grams through the serosal application of 80% acetic acid (see Section 2.2). In pilot studies, we observed considerable variability in the size of ulcers during the first week after application of acetic acid but by day 7, the ulcer size was consistent. For this reason, these studies of the effects of various drugs on ulcer healing were started on the seventh day after induction of ulcers. On the seventh day after induction of ulcers, the rats were weighed, then given one of the following drugs orally (1 mL/kg by gavage): diclofenac (5 mg/kg), nitrofenac (7.5 mg/kg; equimolar to the dose of diclofenac), nabumetone (75 mg/kg), or L745,337 (5 mg/kg). Control rats were treated with the same volume of the vehicle. The test drugs were initially dissolved in dimethyl sulfoxide and then diluted in 1% carboxymethylcellulose such that the final concentration of dimethyl sulfoxide was 5%. The doses of the test drugs were selected because they have been shown to exhibit comparable anti-inflammatory effects (~50% inhibition of carageenan-induced paw edema) in previous studies (76,77,192,196). In addition, the dose of diclofenac was selected because, in previous studies performed in our laboratory, higher doses of this drug resulted in a high rate of mortality due to small bowel perforation when given repeatedly during the period of a week (78). The drugs were given every 24 hours for the next seven days, and the rats were killed 24 hours after the final dose of the drugs. The stomach was removed and pinned onto a wax block. Ulcer area was determined by planimetry (see Section 2.2). All planimetric determinations were performed using coded photographs such that the observer was unaware as to the treatment the rat had received. Sections of the affected region of the stomach were placed in neutral buffered formalin and processed by routine techniques for light microscopy (H&E staining). The slides were coded to avoid observer bias. A blood sample was drawn from a tail vein at the beginning and end of the study for determination of hematocrit. Body weight was measured daily throughout the study.
To confirm that the test compounds were bioavailable after oral administration, separate groups of 5 rats each were treated orally with the test compounds at the same doses as above or with an identical volume (1 mL/kg) of the vehicle. Three hours later, the rats were anesthetized with pentobarbital (65 mg/kg), and a 1-mL blood sample was drawn from the descending aorta. Thromboxane B2 synthesis by the blood was determined (see Section 2.3). Thromboxane B2 is the stable hydration product of thromboxane A2, which is produced by platelets through the enzyme COX-1 (85).

**Effects of a nitric oxide donor.** To determine if an NO-generating compound would alter the healing of gastric ulcers, as has been reported previously (70), we tested the effects of daily oral administration of glyceryl trinitrate or the same volume of the vehicle (water; 1 mL/kg) from day 7 to day 14 after induction of ulcers. Glyceryl trinitrate was tested at the same dose previously reported to accelerate ulcer healing (10 mg/rat) (70) and at lower doses (1 and 0.1 mg/rat). Ulcer area, body weight, and hematocrit were measured as above.

**Effects of Misoprostol.** Rats were treated daily with the prostaglandin E1 analogue, misoprostol (0.01 mg/rat in 0.25 mL per os), from days 7 to 14 after induction of gastric ulcers with acetic acid. As in the above experiments, ulcer area was determined on day 14. Control rats were treated with the same volume of vehicle. This dose of misoprostol was selected because it has previously been shown in studies performed in this laboratory, to prevent the gastropathy that develops in a rat model of portal hypertension secondary to biliary cirrhosis (197). On a per kilogram basis (~50 µg/kg), it is a very similar dose to the 33 µg/kg used by Penney et al. (11), in a study in which they showed accelerated ulcer healing in the rat when co-administered with aspirin over a 2-week period.

**Effects on Acid Secretion.** Any effect of the test drugs on ulcer healing could be attributable to effects on gastric acid secretion because inhibition of acid secretion has been shown to lead to accelerated healing in experimental gastric ulcer models (28,29). We tested the effects of diclofenac (5 mg/kg), nitrofenac (equimolar dose, 7.5 mg/kg), and glyceryl trinitrate (10 mg/rat) on gastric acid
secretion in rats in which an ulcer had previously been induced. We also examined acid secretion in rats with and without a pre-existing ulcer who had then received oral vehicle instead of one of the test drugs. Ulcers were induced as described above. Seven days later, these rats and the group of rats in which ulcers were not induced were deprived of food, but not water, overnight. The rats were given vehicle or one of the test drugs orally. Two hours later, the rats were anesthetized with urethane (25%; 7 mL/kg), a laparotomy was performed and the pylorus ligated. Two hours later, the stomach was excised after clamping the esophagus to prevent leakage of the contents, and the gastric juice was collected into test tubes. Total acid output was calculated (see Section 2.5) and is expressed in units of microequivalents. Each test group consisted of 5 rats.

Statistical analysis. All data are expressed as the mean ± SEM. Comparisons between groups of data were made using a one-way analysis of data followed by a Student-Newman-Keuls post hoc test. An associated probability of <5% was considered significant.

Materials. L745,337 was kindly provided by Dr. C.C. Chan of Merck-Frosst (Montréal, PQ).

4.3 Results

Gastric ulcer healing. Serosal application of acetic acid resulted in the formation of ulcers that persisted for several weeks. Mean ulcer area at day 7 after acetic acid application was 34.1 ± 6.4 mm² (n=10), and 22.5 ± 2.5 mm² (n=40) at day 14 in rats treated only with vehicle.

Daily administration of diclofenac from days 7 to 14 after ulcer induction did not significantly alter ulcer area relative to the vehicle-treated group (Figure 4.1). Histologically, the ulcers in rats treated with diclofenac were indistinguishable from those in rats treated with vehicle. On the other hand, daily treatment with nitrofenac resulted in a significant acceleration of ulcer healing. Ulcer areas were
significantly reduced ($P<0.05$) compared with both the vehicle-treated and diclofenac-treated groups (Figure 4.1). Whereas complete healing of the ulcers was not observed with nitrofenac treatment during a period of one week, ulcer areas were reduced to as little as 1.5 mm$^2$.

Neither diclofenac nor nitrofenac produced macroscopically visible damage to the small intestine or to regions of the stomach other than the site of ulcer induction by acetic acid. However, diclofenac administration resulted in a significant attenuation of the rate of body weight increase compared with the other two groups (Figure 4.2). Moreover, the hematocrit decreased significantly ($P<0.001$) during the course of 7 days of treatment with diclofenac, whereas it was unaffected in the nitrofenac- or vehicle-treated rats (Figure 4.3). These observations suggest that diclofenac did produce significant bleeding, most likely from the small intestine (although bleeding from the gastric ulcer of anemia unrelated to gastrointestinal bleeding cannot be ruled out).

Daily administration of nabumetone or L745,337 also had no effect on the healing of gastric ulcers (Figure 4.4). These drugs had no effect on the rate of body weight gain nor on hematocrit when compared to the vehicle-treated group (data not shown).

Blood thromboxane B$_2$ synthesis was measured to provide an index of the bioavailability of the various test drugs and an index of their effects on COX-1 activity. As shown in Figure 4.4, all of the test drugs significantly inhibited thromboxane B$_2$ production at the doses used. There were no significant differences among the test drugs in terms of their effects on thromboxane synthesis.

Daily treatment with misoprostol failed to significantly alter the rate of gastric ulcer healing (Figure 4.5). However, daily treatment with glyceryl trinitrate resulted in a dose-dependent reduction in the size of gastric ulcers when examined at the end of the one-week treatment period (Figure 4.5). At the highest dose of glyceryl trinitrate tested (10 mg/rat), complete healing of the gastric ulcers (confirmed histologically) was observed in 4 of the 6 rats. Neither glyceryl trinitrate nor
misoprostol significantly affected the rate of weight gain or the hematocrit (data not shown).

**Acid secretion.** To determine if the accelerated healing of ulcers observed when rats were treated with nitrofenac or glyceryl trinitrate was attributable to suppression of gastric acid secretion, experiments were performed to determine if these drugs exerted such effects in pylorus-ligated rats. Acid secretion was significantly higher in rats with acetic acid ulcers that were treated with diclofenac or nitrofenac when compared to rats without ulcers that were treated only with vehicle (Figure 4.6). However, among the groups of rats with pre-existing ulcers, none of the test drugs significantly affected gastric acid secretion.
Figure 4.1. Gastric ulcer area before and after seven days of treatment with test drugs. Seven days after ulcer induction, the mean ulcer area was $34.1 \pm 6.4$ mm$^2$. Following seven days treatment with vehicle, the mean ulcer area was $22.5 \pm 2.5$ mm$^2$ ($P = 0.055$). Daily oral administration of diclofenac (5 mg/kg) did not significantly alter gastric ulcer area, whereas treatment with an equimolar dose of nitrofenac (7.5 mg/kg) significantly reduced gastric ulcer area compared to both the vehicle- and diclofenac-treated groups. Each bar represents the mean $\pm$ SEM of 10 (nitrofenac and diclofenac) or 20 (vehicle) rats. ** $P<0.01$ compared with the vehicle-treated group.
Figure 4.2. Effects of oral administration of vehicle, diclofenac or nitrofenac on changes in body weight. Test compounds were administered daily from day 7 to day 14 following ulcer induction. Nitrofenac (7.5 mg/kg) was administered at an equimolar dose to diclofenac (5 mg/kg). Treatment with diclofenac resulted in a significant depression in body weight gain over the seven-day treatment period, compared with the other two groups. Each group consisted of 10 (diclofenac and nitrofenac) or 20 (vehicle) rats. *P < 0.05, **P < 0.01, ***P < 0.001
**Figure 4.3.** Effects of oral administration of vehicle, diclofenac or nitrofenac on hematocrit. Test compounds were administered daily from day 7 to day 14 following ulcer induction. Nitrofenac (7.5 mg/kg) was administered at an equimolar dose to diclofenac (5 mg/kg). Diclofenac administration significantly reduced hematocrit compared to vehicle- and nitrofenac-treated rats. Each bar represents the mean ± SEM of 10 (diclofenac and nitrofenac) and 20 (vehicle) rats. ***P<0.001.
**Figure 4.4.** Effects of various test drugs on whole blood thromboxane synthesis and gastric ulcer area. Whole blood thromboxane synthesis was measured as an index of COX-1 activity. Drugs were orally administered for one week. Animals were treated with either vehicle (n=40), diclofenac (5 mg/kg; n=10), nitrofenac (7.5 mg/kg; n=10), nabumetone (75 mg/kg; n=10) or L745,337 (5 mg/kg; n=10). Data shown for the vehicle are pooled for all experiments (n=40), however statistical comparisons were made between the test drug and the vehicle-treated rats that were assessed on the same day (n=10-20). **P<0.01, ***P<0.001 compared with the vehicle-treated group. There were no significant differences among the test drugs.

(figure on following page)
Figure 4.4. Effects of various test drugs on blood thromboxane synthesis (top) and gastric ulcer area (bottom).
Figure 4.5. Gastric ulcer area before and after treatment with vehicle, glyceryl trinitrate (GTN) or misoprostol (Miso). Gastric ulcers were induced through the serosal application of acetic acid. Seven days after ulcer induction the rats began daily oral treatment with vehicle, GTN (at 0.1, 1 or 10 mg/rat; mean weight ~225 grams/rat), or misoprostol (0.01 mg/rat; mean weight ~225 grams/rat). GTN administration at doses of 1 and 10 mg/rat significantly accelerated gastric ulcer healing. This effect was dependent on a topical action of GTN, as systemic administration of the same doses of GTN did not affect ulcer healing. Each group consisted of 5-10 rats. * $P<0.05$ compared with the vehicle-treated group.
Figure 4.6. Effects of test drugs on gastric acid secretion. Ulcers were induced through serosal application of acetic acid. Seven days after ulcer induction, diclofenac (5 mg/kg), nitrofenac (equimolar dose, 7.5 mg/kg), glyceryl trinitrate (GTN; 10 mg/rat) or vehicle were orally administered two hours prior to pylorus ligation. Acid secretion was also measured in rats without pre-existing ulcers that were administered vehicle. * $P<0.05$, ** $P<0.01$ compared with the vehicle-treated group without pre-existing ulcers.
4.4 Discussion

The retarding effects of NSAIDs on gastric ulcer healing are thought to be attributable to the suppression of gastric prostaglandin synthesis by these agents and possibly to their topical irritant properties (11,27). Some studies have shown that administration of exogenous prostaglandins, such as misoprostol, can prevent the development of and accelerate the healing of NSAID-induced gastric ulcers (34,35). However, the healing of ulcers by exogenous prostaglandins is impaired if NSAID use is continued (36). As discontinuation of NSAID therapy is often not a viable option, since patients need these drugs to relieve the pain associated with their inflammatory joint disease, there is a need for gastrointestinal-sparing anti-inflammatory agents, particularly for patients prone to ulceration.

In the present study, we have shown that healing of experimental gastric ulcers can be accelerated by an NO-releasing NSAID derivative or by an NO donor (glyceryl trinitrate). A standard NSAID (diclofenac) and two NSAIDs reported to have greater selectivity for COX-2 (96,192) had no effect on ulcer healing in this study. The acceleration of healing observed with the NO-releasing NSAID (nitrofenac) occurred despite the fact that this compound inhibits COX-1 and COX-2 as effectively as the parent drug diclofenac (76,77,195). Determination of thromboxane production by whole blood confirmed that each of the NSAIDs tested in this study significantly suppressed COX-1 activity because platelet thromboxane is mediated through this isofom of the prostaglandin synthase enzyme (85). The acceleration of healing by nitrofenac and glyceryl trinitrate did not seem to be attributable to effects of these agents on gastric acid secretion because neither drug significantly affected rates of acid secretion, at least during the first 4 hours after their administration. It remains a possibility that these drugs exerted a delayed inhibitory effect on acid secretion. The absence of a significant beneficial effect of misoprostol on ulcer healing in the present study is consistent with the observations of Penney et al. (11), who found that a similar dose of misoprostol significantly reduced ulcer size when administered concomitantly with aspirin during a two-week period but not when administered for one-week.
These findings are consistent with the hypothesis that NO is capable of accelerating ulcer healing. Konturek et al. (70) previously showed that glyceryl trinitrate was capable of accelerating ulcer healing using the same model as in the present study. Moreover, they showed that suppression of endogenous NO synthesis led to impaired ulcer healing. Further, it has been reported that topical glyceryl trinitrate promotes the healing of anal fissures and ulcers in patients with Crohn's disease (74). Nitrofenac and other NO-releasing NSAIDs have been shown to have several effects consistent with their releasing NO in vivo and in vitro. For example, plasma nitrate and nitrite levels increase significantly within an hour of administration of these compounds (76,77), gastric blood flow is maintained after administration of an NO-releasing NSAID, whereas it decreases after administration of the native NSAID (77), and leukocyte adherence to mesenteric venules is observed after administration of an NSAID but not the NO-releasing derivative (76). Furthermore, NO-releasing NSAIDs increase intracellular cyclic guanosine monophosphate levels in cultured human umbilical vein endothelial cells (131) and platelets (198).

The mechanisms through which NO promotes ulcer healing are not entirely clear. It is now well established that NO is an important mediator of gastric mucosal defense, modulating mucosal blood flow (199) and mucus secretion (200). Thus, it is possible that NO accelerates ulcer healing by elevating the resistance of the mucosa at the ulcer margin to further damage. Because various growth factors have been shown to modulate ulcer healing (201,202) and the receptors for some growth factors (e.g., epidermal growth factor) are expressed in much greater numbers at sites of ulceration (203), it is possible that NO exerts its effects by modulating the production or action of these factors. It is also possible that NO directly stimulates tissue repair. As well as being shown to promote ulcer healing in the present study and previously (70), NO has been shown to promote the growth of intestinal smooth muscle in a rat model of colitis (204). NO synthesis has also been reported to be an important regulator of wound collagen accumulation and subsequent wound mechanical strength (75). These mechanisms through which NO promotes ulcer healing require further study.
Unlike many previous studies, we did not observe an impairment of ulcer healing in the present study with an NSAID. This difference from previous studies may be related to the different NSAID used because earlier studies used either indomethacin or aspirin (11,28,29), or may be related to the dose of diclofenac we selected, which does not completely suppress gastric prostaglandin synthesis (77), or to the duration of administration of the drug. The selection of the dose of diclofenac used in the present study was based on earlier studies in which significant small intestinal damage was observed with higher doses, frequently leading to perforation and death (78). Even with the dose of diclofenac used (5 mg/kg), we observed a significant depression of body weight gain and a significant decrease in hematocrit. Although we cannot rule out the possibility that this was caused by bleeding from the gastric ulcers or was unrelated to bleeding from the gastrointestinal tract, it seems more likely to be due to small intestinal lesions that were not macroscopically detectable.

It is interesting that the two reportedly selective COX-2 inhibitors, nabumetone and L745,337, did not affect ulcer healing rates in this study. These drugs suppressed COX-1 activity (thromboxane synthesis) as effectively as diclofenac at the doses used. These doses were selected because, in previous studies (96,101,192), they were reported to exert approximately equivalent anti-inflammatory activity (~50% inhibition of carageenan-induced paw edema) to what we observed with diclofenac and nitrofenac at the doses used (77). Thus, although these drugs have been shown to have greater selectivity in vitro for COX-2 (96,192), the doses required for anti-inflammatory activity were found in the present study to be sufficient to significantly inhibit COX-1. It is not possible to accurately measure prostaglandin production from COX-2 in an in vivo system as we did for COX-1 through the measurement of thromboxane production. However, given the greater potency of nabumetone and L745,337 as inhibitors of COX-2, it is reasonable to assume that this enzyme was markedly suppressed by doses that suppressed COX-1 activity. These data therefore suggest that suppression of COX-2 does not lead to impairment of gastric ulcer healing, at least at the doses of the test drugs utilized in this study. However, these results are in contrast to those
obtained by Schmassmann et al. (107). They (107) observed significant inhibition of gastric ulcer healing with L745,337, with an ID_{50} value of 1.7 mg/kg. This discrepancy in findings may be explained by differences in study design. COX-2 is rapidly induced, as early as 1 hour following an ulcerogenic stimuli (109). Elevated levels of COX-2 mRNA expression have been shown to peak between day 3 and day 5 following ulcer induction and thereafter gradually decrease with time (105,107). Schmassmann et al. (107) began oral administration of the COX-2 inhibitor one day following ulcer induction, a time point consistent with significantly elevated levels of COX-2 mRNA. In addition, the drug was administered twice-daily (107). This is in contrast to our study in which the drug was administered only once a day, and treatment began on day seven following ulcer induction. The possibility exists that drug administration in our study began at a point when the prostaglandins generated by COX-2 were not making as significant a contribution to ulcer healing, thus we did not observe impaired healing. Alternatively, it is possible that the once a day administration of the COX-2 inhibitors in our study was not sufficient to get 24-hour suppression of COX-2, and so healing was not impaired. Whether or not selective inhibitors of COX-2 will influence ulcer healing in a chronic setting of significant gastritis, as would occur with Helicobacter pylori infection, remains to be determined.

In summary, the present study shows the ability of an NO-releasing derivative of a commonly used NSAID to promote the healing of experimental gastric ulcers. The confirmation and extension of the previous report (70) that an NO donor can similarly promote ulcer healing suggest that the acceleration of healing by the NO-releasing NSAID is attributable to the generation of NO from this compound. Further studies are required to better understand the mechanism(s) through which NO modulates ulcer healing.
CHAPTER 5

BACTERIA RAPIDLY COLONIZE AND DELAY THE HEALING OF GASTRIC ULCERS IN RATS

5.1 Introduction

The stomach and upper intestine are normally sterile, with low levels of flora only being present for a short period of time after meals. A notable exception is the colonization by Helicobacter pylori of the upper gastrointestinal tract of a significant portion of the human population. This bacterium is specially adapted to survive in the low-pH environment of the stomach through its ability to generate a local microenvironment with a more tolerable pH (30).

An association between bacterial colonization of the stomach and gastric ulcers was suggested more than a century ago (115,128). Considerable data have been reported to support such an association. For example, Saunders (120) isolated an α-Streptococcus from peptic ulcers in humans, whereas Letulle (116) demonstrated that oral or parenteral administration of Staphylococcus pyogenes to guinea pigs resulted in the development of gastric ulcers. However, a clear consensus on the contribution of these bacteria to ulceration was never reached, and since the discovery of the association between H. pylori colonization and peptic ulcer disease, the possible contribution of other bacteria to this disease has essentially been supplanted. It remains possible, however, that bacteria other than those of the Helicobacter genus could contribute to gastric ulceration if they were capable of colonizing the stomach, or more specifically, the ulcer site. In experimental models, bacteria appear to play an important role in exacerbating mucosal injury induced in the stomach or small intestine by nonsteroidal anti-inflammatory drugs (76,131,132).

In the present study, we tested the hypothesis that bacteria selectively colonize sites of ulceration in the rat stomach and have a negative impact on the
healing of those ulcers. In particular, the questions that were asked in this study were as follows. 1) How rapidly does bacterial colonization occur following ulcer induction? 2) Are certain species of bacteria more prevalent than others? 3) Is bacterial colonization specifically localized to the ulcer or is it more widespread? 4) Do changes in gastric acid secretion occur that are permissive to bacterial colonization of the stomach? 5) Can we manipulate the species of bacteria colonizing the stomach and, in doing so, influence the rate of gastric ulcer healing?

5.2 Materials and Methods

Histological evaluation of gastric ulcers. In order to evaluate histologically the ulcer site for damage and the presence of bacteria, ulcers were induced in male, Wistar rats (see Section 2.2). Seven days after ulcer induction, the rats were killed by cervical dislocation. The stomach was removed, rinsed with sterile PBS (pH 7.4), pinned out on a wax block, and submerged in Carnoy’s fixative (ethanol:chloroform:glacial acetic acid, 6:3:1) for 2-4 hours. Tissue samples were taken from the ulcer bed, placed in a histology cassette and then transferred to 50% ethanol. Tissue samples were also taken from a group of control rats, or rats with no ulcers, for comparison. Tissue samples of the ulcers and healthy tissue were embedded in plastic using a commercially available kit (JB-4 Embedding Kit, Polysciences, Warrington, PA). Sections of 1-1.5 μm in thickness were cut, mounted on glass microscope slides, and heat-fixed for approximately one hour at 50°C. The sections were stained by immersion for approximately ten seconds in a methylene blue-basic fuchsine solution (BDH, Edmonton, AB, Canada). The microscope slides were rinsed with distilled water, allowed to air dry, and coverslips applied using CytosealTM XYL (Stephens Scientific, VWR; Edmonton, AB, Canada). Bacteria, identified as either rods or cocci, were visualized by light microscopy.
**Bacterial culturing of gastric ulcers.** To determine the time course of bacterial colonization of acetic acid-induced gastric ulcers, rats were killed at various time points after ulcer induction (1 hour-21 days). The stomachs were removed and tissues taken for bacterial culturing (see Section 2.4). Bacterial levels found in the ulcer bed and contralateral tissues were expressed as colony forming units (CFU) per gram of gastric tissue. To eliminate the possibility that bacteria were being introduced to the rats during the ulcer-induction procedure, a group of rats underwent sham ulcer induction (see Section 2.2). Seven days after the sham ulcer induction, the rats were killed by cervical dislocation, and tissue samples were taken from the corpus region of the stomach at the approximate site where the barrel of the syringe was placed for the sham induction. These tissue samples were processed and underwent bacterial culturing (see Section 2.4) for determination of bacterial levels.

**Effect of coprophagy and feeding on bacterial colonization.** The potential exists that bacteria may be introduced into the rat's gastrointestinal tract via the ingestion of food or fecal matter (coprophagy). To determine the influence of coprophagy and feeding on bacterial colonization of gastric ulcers in rats an experiment was performed in which acetic acid gastric ulcers were induced in three groups of rats (see Section 2.2) and the levels of bacterial colonization were assessed 18 hours later. One group of rats was housed in standard cages, and the rats were not fasted. The other two groups were housed in wire mesh-bottomed cages (to reduce coprophagy). Both groups were fasted before ulcer induction, and in one of these groups the fasting continued for the 18-hour period after ulcer induction.

**Effect of ulcer induction model on bacterial colonization.** In order to determine if the bacterial colonization of gastric ulcers was unique to the acetic acid model, gastric ulcers were induced in rats by two other methods: cryostatically, via the application to the serosal surface of a cryoprobe, and with the nonsteroidal anti-inflammatory drug, naproxen (antral ulcer model).

**Cryoprobe ulcer induction.** Rats were fasted overnight with free access to drinking water. Under halothane-induced anesthesia (5% v/v), a midline
laparotomy was performed and the stomach gently exteriorized. The base of a glass cuvette (7.65 mm in diameter), which had been allowed to equilibrate in liquid nitrogen and was about one-fourth full of liquid nitrogen, was placed on the serosal surface of the stomach at approximately the centre of the corpus. The cuvette remained in contact with the stomach for 30 seconds, after which time it was refilled and the process repeated twice. The abdominal incision was closed and the rat allowed to recover with free access to laboratory chow and drinking water. Seven days after ulcer induction, the rats were killed by cervical dislocation and tissue samples of the ulcer sites taken for bacterial culturing (see Section 2.4).

**Antral ulcer model.** A slightly modified version of the re-fed rat model of acute antral ulcers, originally described by Satoh et al. (205), was used. Rats were deprived of food, but not water for 18-24 hours. At the end of this period, food was returned for 2 hours. Following this re-feeding, the rats were orally dosed with either vehicle (5% sodium bicarbonate; 2 mL/kg) or naproxen (80 mg/kg; 2 mL/kg). The rats were then fasted a further 24 hours. At the end of this second fasting period, the rats were killed by cervical dislocation. Tissue samples of the ulcers, or corresponding areas from vehicle-treated rats, were taken for bacterial culturing (see Section 2.4).

**Identification of the bacterial species present.** Ulcers were induced in rats by the serosal application of acetic acid (see Section 2.2). Tissue samples of gastric ulcers were collected from rats killed 1, 7, and 14 days after ulcer induction. Samples were also taken from the corresponding region of the stomach of healthy controls. The samples were processed for bacterial culturing (see Section 2.4) and following determination of bacterial levels, the MacConkey and trypticase soy agar plates were transferred to the Provincial Microbiology Laboratory (Foothills Hospital, Calgary, AB, Canada) for detection and identification of the bacterial species. The basis of the tests for the detection and identification of the bacterial species was the ability of the bacteria to form colonies in the presence of a variety of antibiotics (206). The prevalence of each bacterial species was scored on a 0 to 4 scale as follows:
<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
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<tbody>
<tr>
<td>0</td>
<td>no detectable colonization</td>
</tr>
<tr>
<td>1</td>
<td>scarce colonization</td>
</tr>
<tr>
<td>2</td>
<td>light colonization</td>
</tr>
<tr>
<td>3</td>
<td>moderate colonization</td>
</tr>
<tr>
<td>4</td>
<td>heavy colonization</td>
</tr>
</tbody>
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These values represent the amount of growth by a specific bacterial species on an agar plate, where a value of 1 is equivalent to growth in one quadrant, 2 in two quadrants, etc. These assessments were made by an observer unaware of the treatments the rats had received. The scores from each rat in a group were summed and a bacterial colonization index was calculated, which consisted of the percentage of the maximal colonization (i.e., 100% colonization corresponded to a score of 4 in all rats in the group).

**Effects on gastric acid secretion.** Gastric ulcer healing may be influenced by inhibition of acid secretion as it has been shown to lead to accelerated healing in experimental gastric ulcer models (29). The presence of gram-negative bacteria in the stomach may, through the liberation of endotoxin, influence acid secretion (207) and therefore the rate of ulcer healing. To examine these possibilities, a continuous gastric perfusion system was used to measure gastric acid output (see Section 2.5) in rats 1, 3 and 10 days after ulcer induction. Gastric acid output was also measured in control animals that did not have pre-existing ulcers.

In order to determine if there was an alteration in the pH at the ulcer site which might facilitate bacterial colonization, Congo red dye (Sigma Chemical Co., St Louis, MO) was utilized. Congo red (25 mg/mL; 1 mL; solubilized in sterile saline) was orally administered to rats 1 or 7 days after ulcer induction with acetic acid (see Section 2.2). Congo red is a pH-sensitive dye that appears red at a pH >5 and blue at a pH <3 (208). Thirty minutes after dosing, the rats were killed and the stomach removed. The stomach was cut along the greater curvature, rinsed with sterile saline, pinned out on a wax block and photographed for subsequent evaluation by an observer unaware of the treatments the rats had received.
**Effect of antibiotic administration.** The effects of daily treatment with the broad-spectrum antibiotics streptomycin and penicillin were determined. Ulcers were induced in the rats with acetic acid (see Section 2.2). Seven days after ulcer induction, a 7-day treatment regimen was initiated during which streptomycin (168 mg/mL; 0.5 mL; Sigma Chemical Co., St. Louis, MO) or penicillin (84 mg/mL; 0.5 mL; Sigma Chemical Co., St. Louis, MO) was orally administered twice daily. Control rats received an equivalent volume (0.5 mL) of vehicle (distilled water). On the fourteenth day after ulcer induction, the rats were killed by cervical dislocation, the stomach was removed for ulcer area determination (see Section 2.2) and tissue samples were taken for bacterial culturing (see Section 2.4). In a separate experiment, the effect of treatment with the combination of streptomycin (336 mg/mL; 0.25 mL) and penicillin (168 mg/mL; 0.25 mL) was assessed. Control rats received an equivalent volume (0.5 mL) of vehicle (distilled water). Ulcers were induced in the rats with acetic acid (see Section 2.2) and on the seventh day after ulcer induction, rats began twice daily oral dosing with the combination of antibiotics or vehicle. Seven days later (i.e., fourteen days after ulcer induction), the rats were killed, the stomach removed for ulcer area assessment (see Section 2.2) and tissue taken for bacterial culturing (see Section 2.4). The doses of streptomycin and penicillin used were based on a study by Deitch et al. (209), who showed them to be effective at significantly reducing cecal aerobic and facultatively anaerobic bacterial levels.

**Effect of selective colonization with Eschericia coli.** This study was performed to determine the impact of selective colonization of the gastric ulcer with a gram-negative bacterium on ulcer healing. Ulcers were induced through the serosal application of acetic acid (see Section 2.2). Immediately thereafter, the rats received a combination of the antibiotics, bacitracin (4 mg/mL; Sigma Chemical Co., St. Louis, MO) and streptomycin (4 mg/mL; Sigma Chemical Co., St. Louis, MO), in their drinking water. On the third day after ulcer induction, one group of rats began oral dosing with 1 mL of MacConkey broth (Becton-Dickinson, Cockeysville, MD) containing $10^9$ CFU of a streptomycin-resistant strain of *E. coli* C-25 (210). This treatment was repeated every 12 hours for 7 days. A control
group of rats received 1 mL of the MacConkey broth orally at the same time. The rats in the control and treatment groups were housed separately so as to avoid ingestion of *E. coli* C-25 by coprophagy. On the tenth day after ulcer induction, the rats were killed by cervical dislocation. Half of the rats from each treatment group had their stomachs removed and pinned out for ulcer area determination (see Section 2.2). Tissue samples of the ulcer sites and contralateral sites were taken from the remaining rats in each group for determination of the degree of bacterial colonization (see Section 2.4).

**Effect of lactulose administration.** It has been suggested that lactobacillus, a gram-positive bacterium, is beneficial in the gastrointestinal tract. Lactulose (4-O-β-D-galactopyranosyl) has been shown to promote the growth of lactobacilli, especially *Lactobacillus acidophilus* (211). This study was performed to determine if the addition of lactulose (5% vol/vol; Lactulax, Pharmascience, Montréal, PQ, Canada) to the drinking water would influence the gastric levels of gram-positive bacteria and if this would subsequently alter the rate of ulcer healing. The rats were divided into four groups. One group received lactulose in the drinking water for 2 weeks and were then killed. Gastric tissue samples were taken from these rats for bacterial level determination (see Section 2.4). A second group of rats received lactulose for 2 weeks prior to ulcer induction with acetic acid and continued to receive it for 10 days thereafter. A third group of rats received the 5% lactulose solution in the drinking water for days 3 through 10 after ulcer induction. Control rats, which had ulcers, received regular drinking water throughout the experiment. On the tenth day after ulcer induction, the rats were killed, and ulcer area and bacterial colonization levels were determined (see Sections 2.2 and 2.4, respectively).

**Statistical analysis.** Comparisons among groups of data were made using a one-way ANOVA and a Newman-Keuls test. For comparisons of bacterial colonization at ulcer sites vs. contralateral sites, a paired Student's *t*-test was used. With all analyses, *P* <0.05 was considered significant.
Materials. *E. coli* C-25 was generously provided by Dr. E. Deitch of New Jersey Medical School (Newark, New Jersey).

5.3 Results

**Bacterial colonization of gastric ulcers.** Histological examination of the ulcer bed revealed considerable levels of colonization by a variety of bacteria. Both rods and cocci were evident. Bacterial colonization of adjacent, normal tissue was occasionally observed, but appeared to be much less dense than that seen in the ulcer bed. In these cases, the bacteria were restricted to the luminal surface of the epithelium and no invasion into the mucosa or glands was observed.

Culturing of tissues taken from rats with ulcers confirmed the extensive colonization of the ulcer bed by various bacteria. In the stomachs of rats without ulcers, the levels of total aerobes ranged from $10^3$ to $10^4$ CFU/g tissue. Gram-negative bacteria constituted a small portion (approximately 5%) of total aerobes. Significant colonization of the stomach occurred as early as six hours after ulcer induction. The levels of total aerobes colonizing the ulcer bed increased to a peak level of $\sim10^9-10^{10}$ between 12 and 24 hours after ulcer induction and remained at or near those levels for the following 7 days (Figure 5.1). Gram-negative bacteria accounted for the majority of total aerobes during this period (Figure 5.1). Bacterial levels then gradually declined, with levels at fourteen days after ulcer induction being not significantly different from those in the normal rat stomach. As the numbers of total aerobes declined, the preponderance of gram-positive bacteria was once again evident.

The histological studies suggested that the bacterial colonization was occurring principally at the ulcer site. This was confirmed by bacterial culturing, with significantly greater numbers of bacterial colonies ($\sim10,000$-fold increase in CFU) found in the ulcer bed than on the contralateral side of the stomach (Figure 5.2). In some rats with ulcers, there were also increased numbers of bacteria on the contralateral side relative to rats without ulcers. Bacterial culturing of tissues
taken from animals that underwent sham ulcer induction revealed that the induction procedure itself was not responsible for the introduction of bacteria, as neither total aerobes nor gram negative bacterial levels were different relative to a control group in which ulcers were not induced (Figure 5.3).

Sensitivity testing revealed that the predominant bacteria at day 1 after ulcer induction were *E. coli*, *Streptococcus*, and *Enterococcus* (Figure 5.4). *Lactobacillus*, which was present in the stomach of all healthy controls, was absent one day after ulcer induction. By day 7, *E. coli* was still one of the major bacterial species present, *Enterococcus* levels had increased, and *Streptococcus* had declined. By day 14 after ulcer induction, a point at which healing had reduced the mean ulcer size by ~65% from seven days prior, *E. coli* was still evident but *Streptococcus* and *Enterococcus* were rare. Most notable, however, was the preponderance of *Lactobacillus* colonizing the ulcer site at this time point.

To determine if bacterial colonization was a feature unique to ulcers induced by acetic acid, ulcers were induced using two other methods: the application of a cryoprobe or by naproxen administration. Both alternative methods of ulcer induction resulted in a marked increase in gastric ulcer colonization by aerobes compared with a control group of rats that had not undergone ulcer induction (Figure 5.5).

Examining the results of the effects of food and coprophagy on bacterial colonization revealed that in all three groups, significant increases in bacterial colonization of the ulcers occurred relative to the levels seen in the healthy rat stomach. In the two groups that were allowed access to food immediately after ulcer induction, the levels of total aerobes in the stomach were significantly higher than in the group that continued to be fasted (~10^9 vs ~10^8 CFU/g; P<0.05). In the latter group, the fasting condition was confirmed in that the stomach was empty at the time of death. Thus it appeared that fasting reduced the levels of colonization of gastric ulcers somewhat but even when food was withheld and steps taken to prevent coprophagy, marked colonization of gastric ulcers still occurred.

**Changes in acid secretion.** Administration of congo red to rats killed 1 or 7 days after ulcer induction resulted in dark blue staining of all tissue with the
exception of the ulcer itself, which stained pink. This differential staining indicated that the surface of the normal gastric tissue had a pH of <3, while the ulcer bed had a pH of >5. This suggested that there may be an impairment in acid secretion and that an environment conducive to bacterial colonization may exist at the ulcer site. To further investigate potential changes in gastric acid secretion associated with the presence of an ulcer, in vivo perfused stomach preparations were used in which basal and pentagastrin-stimulated acid secretion were measured (see Section 2.5). On day 1 after ulcer induction, no effect was observed on either basal or pentagastrin-stimulated acid secretion relative to control rats, or rats with no ulcers (Figure 5.6). However, on day 3 after ulcer induction, a significant suppression of pentagastrin-stimulated acid secretion (~55%) was observed. On day 10 after ulcer induction, gastric acid secretion had returned to levels that were not significantly different from those observed in controls. Planimetric assessments of the area of the gastric ulcer relative to the total stomach area available for acid secretion (the corpus region) revealed that the reduction in acid secretion observed at day 3 was not due solely to a reduction in the area of gastric corpus available for secretion.

Effects of broad spectrum antibiotics on ulcer healing and bacterial colonization. Oral administration for one week of streptomycin or penicillin alone did not significantly affect the level of total aerobic bacterial colonization at the ulcer site (Figure 5.7). Treatment with these antibiotics individually also did not affect the healing rate of the gastric ulcers. However, when these antibiotics were used in combination, a marked reduction (~68%) in the extent of bacterial colonization was observed and this was accompanied by a significant acceleration of ulcer healing relative to the vehicle-treated control group (Figure 5.7).

Selective colonization of gastric ulcers with E. coli C-25. Rats receiving bacitracin and streptomycin in the drinking water and treated daily for a week with bacteria-free MacConkey broth had significantly lower levels of bacterial colonization of their ulcers and significantly enhanced ulcer healing compared to untreated control (Figure 5.8). In contrast, rats receiving bacitracin and
streptomycin in the drinking water but treated twice-daily with the streptomycin-resistant strain of *E. coli* (C-25) had significantly higher levels of bacterial colonization and significantly larger gastric ulcers relative to the group receiving the culture broth (Figure 5.8). In fact, in the rats receiving the antibiotics and *E. coli* C-25, the levels of bacterial colonization and mean ulcer size did not differ significantly from those in untreated controls. Colonization with *E. coli* C-25 did occur preferentially at the ulcer site, as confirmed by bacterial cultures taken from the ulcer and a contralateral site in the stomach (Figure 5.9).

**Effects of lactulose administration.** The addition of 5% (v/v) lactulose to the drinking water for 2 weeks led to a significant increase in total aerobes in the stomach (~40-fold). This increase appeared to be at the expense of the gram-negative bacteria, as they were almost completely eradicated (Figure 5.10). In rats in which lactulose had been given for 2 weeks, ulcers induced, and lactulose administration continued for 10 days, gastric ulcer size was significantly reduced compared to rats that had received regular drinking water throughout the study. Treatment with lactulose only from day 3 to day 10 after ulcer induction did not significantly affect ulcer size.
Figure 5.1. Bacterial colonization of gastric ulcers in rats. Levels of total aerobes and gram-negative bacteria are shown. Changes in bacterial levels over the first 24 hours (top) and during the period of days 1-21 (bottom) following ulcer induction are shown. The solid lines indicate the mean number of total aerobes in healthy controls, while the dashed line represents the mean number of gram-negatives in healthy controls. Note that the ordinate is a logarithmic scale. Each group consisted of at least 5 rats.
Figure 5.2. Bacterial colonization of gastric ulcers vs. the contralateral, normal side of the rat stomach. Total aerobe CFU for 6 rats killed on the seventh day after ulcer induction are shown. Significantly greater levels of bacterial colonization were found at the ulcer site compared with the contralateral side of the stomach. The box represents the mean ± SEM of the level of total aerobes found in healthy rats with no ulcers. The mean level of bacterial colonization found on the contralateral side of the stomach did not differ significantly from levels found in healthy controls. *** P<0.001 compared with bacterial levels at the ulcer site.
**Figure 5.3.** Levels of bacterial colonization from healthy controls (no ulcers) and rats undergoing sham ulcer induction. Total aerobes and gram-negative bacteria are shown. Sham ulcer induction did not significantly increase levels of either total aerobes or gram-negative bacteria.
Figure 5.4. Species of bacteria colonizing gastric ulcers in rats before and 1, 7, and 14 days after their induction. The prevalence of each bacterial species found in cultures taken from the ulcer site was scored on a scale of 0 to 4 as follows: 0, no detectable colonization; 1, scarce colonization; 2, light colonization; 3, moderate colonization; and 4, heavy colonization. The scores from each rat in a group were summed and a bacterial colonization index was calculated that consisted of the percentage of maximal colonization observed. Each group consisted of at least 5 rats.
Figure 5.5. Effects of different methods of gastric ulcer induction on levels of total aerobes colonizing the ulcer site. Ulcers were induced with acetic acid, naproxen administration (NSAID ulcer), or with cryoprobe application (cryo ulcer). Bacterial colonization was significantly increased with ulcer induction by all three methods. The mean levels of total aerobe bacterial counts did not differ significantly among the different induction methods. ** $P<0.01$ compared to healthy controls with no ulcers.
**Figure 5.6.** Effects of ulcer induction on acid secretion in the rat. Basal and pentagastrin-stimulated acid secretion were measured in rats before, 1, 3, and 10 days after ulcer induction. **P<0.01** compared with corresponding control data. Each group consisted of at least 5 rats.
Figure 5.7. Effects of streptomycin and penicillin, alone or in combination, on gastric ulcer area (bottom) and levels of total aerobes colonizing the gastric ulcer (top). Drugs were orally administered twice-daily for one week, beginning on the seventh day after ulcer induction. * P<0.05, ** P<0.01 compared with vehicle-treated rats.
Figure 5.8. Effects of a streptomycin-resistant strain of *E. coli* on gastric ulcer healing (*bottom*) and on the levels of total aerobes colonizing the ulcer site (*top*). Immediately after ulcer induction, rats began to receive streptomycin and bacitracin in their drinking water for 10 days. One group of rats received $10^9$ CFU of *E. coli* C-25 orally each day from days 3 to 10, while the other group received bacteria-free culture broth during the same period. *** $P<0.001$ compared with the untreated group of rats killed at the same time (day 10). $\delta$ $P<0.05$, $\delta\delta$ $P<0.01$ compared with the group receiving antibiotics + culture broth. Each group consisted of at least 5 rats.
Figure 5.9. Bacterial colonization with *E. coli* C-25 of gastric ulcers vs. contralateral, normal side of the stomach. Total aerobe CFU for 5 rats killed on day 10 after oral administration of the streptomycin-resistant strain *E. coli* C-25. There were significantly greater numbers of bacteria at the ulcer site than on the contralateral side of the stomach, indicating selective colonization of the ulcer site by *E. coli* C-25. The box indicates the mean ± SEM of the number of total aerobes in healthy controls (no ulcers). The mean levels of total aerobe bacterial counts on the contralateral side of the stomach did not differ significantly from those in healthy controls. **P<0.01 compared with bacterial levels at the ulcer site.
Figure 5.10. Effects of the addition of 5% lactulose to the drinking water on bacterial colonization of the ulcer site (top) and healing of gastric ulcers (bottom). Rats were given lactulose-supplemented drinking water (or regular drinking water) for 2 weeks. Tissues for assessment of bacterial colonization were taken at the end of the 2-week period. In other rats, ulcers were induced and lactulose administration continued for an additional 10 days. Ulcer areas were determined on the tenth day. *P<0.05 compared with the group receiving only water. Each group consisted of at least 5 rats.
5.4 Discussion

Within a few hours of ulcer induction, the stomach of the rat is changed from an environment that is unsuitable for significant bacterial colonization to one in which profound colonization occurs. This colonization occurred principally at the site of the ulcer. Colonies were found within the cellular debris and mucus overlying the ulcer bed, a site of relatively high pH (>5) compared with the surface of normal tissue (pH<3). While gram-positive bacteria accounted for ~95% of the bacteria found in the stomach of normal rats, with \textit{Lactobacillus} being the predominant variety identified, ulcer induction resulted in a rapid shift to a preponderance of gram-negative bacteria. No single bacterial species predominated in the early period after ulcer induction. As the ulcer healed, however, the numbers of total aerobes and the proportion of the total made up of gram-negative bacteria declined. As was the case in the healthy rats, \textit{Lactobacillus} was the predominant bacteria colonizing the stomach at 21 days after induction of ulcers.

The presence of bacteria at the site of ulcers in the stomach clearly affected the natural history of the ulcer in that reduction of bacterial numbers with antibiotic administration led to a significant acceleration of ulcer healing. Moreover, during broad spectrum antibiotic therapy, inoculation with an antibiotic-resistant strain of \textit{E. coli} (C-25) delayed ulcer healing.

A beneficial effect of gram-positive bacteria with respect to ulcer healing was suggested by the appearance of \textit{Lactobacillus} during the period when there was a diminution of gram-negative bacteria and when substantial ulcer healing occurred (\textit{days 7-14}). This hypothesis was further supported by the observation that induction of \textit{Lactobacillus} within the stomach through the addition of lactulose to the drinking water occurred at the expense of gram-negative bacteria and significantly reduced gastric ulcer area. Lactobacilli have previously been shown to prevent infection of the stomach of gnotobiotic mice with \textit{H. pylori} (137). Based on their findings, Kabir et al. (137) suggested “the possibility of
lactobacilli being used as probiotic agents against *H. pylori*. The ability of probiotics such as lactobacilli to reduce injury in the gastrointestinal tract (138) and inhibit the growth of potentially pathogenic bacteria is well documented and has been attributed to a number of possible mechanisms, including competition for nutrients, production of antimicrobial substances, and stimulation of immunity (212).

A key question raised by the findings of the present study is: What is the nature of the change that occurs in the stomach within hours of ulcer induction that permits colonization? Although the answer to this question is not yet clear, several possibilities exist. First, the region over the ulcer bed that was colonized by the bacteria represented a microenvironment of relatively high pH that would be more conducive to the survival of bacteria. Second, this microenvironment would presumably be rich in nutrients necessary to support bacterial growth. Third, it is possible that the materials overlying the ulcer bed provided an appropriate template for bacterial adherence. It is noteworthy that bacterial colonization occurred irrespective of the method used to induce ulceration. Furthermore, rapid bacterial colonization occurs at sites of injury elsewhere in the gastrointestinal tract. For example, Reuter et al. (213) observed a marked increase in bacterial numbers in the small intestine after induction of injury with diclofenac. This increase in bacterial load occurred subsequent to significant increases in intestinal permeability. As in the case of gastric ulcers, bacteria appear to contribute to the injury induced in the small intestine by nonsteroidal anti-inflammatory drugs, because a reduction of the severity of injury could be observed after treatment with antibiotics (214).

Another key question raised by this study is: How do the bacteria that colonize gastric ulcers interfere with ulcer healing? The increased preponderance of gram-negative bacteria during the hours after ulcer induction and the beneficial effects observed after the induction of a gram-positive bacteria (*Lactobacillus*) suggest that endotoxin may be contributing to the chronicity of the ulcers. Another possibility we considered was that bacterial colonization of the stomach might increase acid secretion. Indeed, it has been suggested that *H. pylori* may
contribute to the pathogenesis of duodenal ulceration by increasing gastric acid secretion (215). Beneficial effects of inhibitors of acid secretion have been documented in this ulcer model (29). However, acid secretion actually decreased on day 3 after ulcer induction (a time when colonization was at peak levels) and normalized thereafter. This observation is consistent with those of others using the same model of gastric ulcer in rats (125,216).

The vast majority of recent research related to bacterial colonization of the stomach is focused on *H. pylori*, and this is justified given the overwhelming evidence that infection of the stomach by this bacterium is a major risk factor for peptic ulcer disease and possibly other disorders (217). There is good evidence that *H. pylori* is specially adapted so that it can survive in the harsh environment of the stomach, as well as evidence that some strains of *H. pylori* carry virulence factors that may promote ulceration (125,126). It is not clear if pre-existing injury to the gastric mucosa is a permissive factor for colonization of the stomach by *H. pylori*, in the manner that induction of gastric ulcers in the rats allows for rapid bacterial colonization, although this has been previously suggested (218,219). However, studies performed in the rat have demonstrated that, while *H. pylori* is not able to colonize or injure the normal stomach, it could colonize the stomach if given to rats twice-daily for a week after an ulcer had been induced (129). Moreover, both intact *H. pylori* and bacteria-free filtrates were capable of delaying gastric ulcer healing in rats (129). These results have been extended by Li et al. (130), who showed a similar delay of ulcer healing in rats inoculated with a vacA⁻ and cagA⁻ strain of *H. pylori*. The results of the present study demonstrate that these effects are not unique to *H. pylori*, because the bacteria that spontaneously colonized the ulcerated stomach and a streptomycin-resistant strain of *E. coli* were capable of significantly delaying ulcer healing. Thus, although *H. pylori* is undoubtedly the major microbial culprit in the pathogenesis of peptic ulcer disease, the results of the present study suggest that other bacteria (and possibly other microbes) are capable of influencing the natural history of ulcers. In this regard, it is noteworthy that *Streptococcus* and *Candida* have been reported to colonize human gastric ulcers and to contribute to the chronicity of those lesions
Although the eradication of *H. pylori* has a profound effect on the healing and the recurrence of ulcers, one cannot exclude the possibility that some of the beneficial effects of treating peptic ulcer disease with wide spectrum antibiotics may be attributable to eradication of bacteria other than *H. pylori*.

In summary, the present study demonstrates a rapid colonization of gastric ulcers in rats by a variety of bacteria. This colonization occurs predominantly at the ulcer site and has a clear detrimental effect in terms of the healing of the ulcer. Gram-negative bacteria are likely to be responsible for the observed delay in ulcer healing, whereas gram-positive bacteria may actually promote ulcer healing. These studies suggest that bacteria other than *H. pylori* have the capacity to significantly influence the natural history of an ulcer and that ulcers represent an environment conducive to bacterial growth.
CHAPTER 6

BACTERIAL COLONIZATION AND GASTRIC ULCER HEALING: THE EFFECT
OF EPIDERMAL GROWTH FACTOR ADMINISTRATION

6.1 Introduction

Epidermal growth factor (EGF) is a 53 amino acid polypeptide that is synthesized in the salivary glands, kidney, duodenal Brunner's glands, pancreas, and lactating mammary glands (143,220,221). Large amounts of EGF may be found throughout the lumen of the gastrointestinal tract (12). Luminal EGF secretion may occur as a result of mucosal injury, as Wright et al. (222) identified a novel ulcer-associated cell lineage that secretes EGF and is induced in the presence of mucosal injury.

EGF mediates its biological effects via binding to a specific 170-kilodalton membrane-bound glycoprotein receptor, the EGF receptor. The EGF receptor has been found throughout the fetal and adult gastrointestinal tract, liver and pancreas (164,223). Binding of EGF to the EGF receptor activates the intrinsic tyrosine kinase, which then leads to a complex cascade of cellular events that ultimately result in DNA synthesis and cellular growth (220). Chronic administration of EGF produces a significant increase in mucosal DNA, RNA and protein content (224). This proliferative action of EGF is believed to contribute to the normal maintenance of mucosal integrity within the gastrointestinal tract (221). EGF has also been shown to be beneficial in pathophysiologic processes in the gastrointestinal tract by either reducing injury (162,201,225-227) or accelerating repair (222,226,228-233). EGF administration is capable of providing protection against a variety of gastric insults, both acid-dependent (162,201) and -independent (160,162,226). The importance of EGF in ulcer healing is demonstrated by the marked increase of EGF receptors and EGF-producing cells around experimental gastric ulcers in rats induced by acetic acid (203,229,234) or cryoprobe (235). Further, the removal of submandibular salivary glands in rodents significantly reduces the rate of healing of
experimentally-induced gastric ulcers, and exogenous administration of EGF to these animals restores healing (229-231). In addition, skin wounds in mice heal more slowly when the animals are caged individually than when the mice are caged in groups where communal licking occurs. Salivary gland extirpation in these animals retarded wound closure and the topical application of EGF to these wounds accelerated healing (232,233). EGF also aids in the restoration of epithelial absorptive function following injury by stimulating an increase of epithelial brush-border surface area (170), electrolyte and nutrient transport (171), phospholipid synthesis (172), and glucose (173), galactose (174) and glycine (174) uptake.

It is well recognized that EGF is critical to mucosal protection and repair by decreasing acid secretion (151), increasing gastric blood flow (160,236), improving restitution (228,237), increasing both the synthesis and secretion of mucus (164,225), and stabilizing the actin cytoskeleton (170,228,238,239). Recent work has demonstrated that EGF may also play a protective role in the GI tract by preventing bacterial colonization of healthy intestinal mucosa (178). EGF is able to decrease the incidence of burn-induced bacterial translocation in mice (176). Liu et al. (177), using a model of acute pancreatitis in rats, demonstrated that EGF treatment could also significantly decrease bacterial translocation while restoring and/or maintaining intestinal mucosal structure and function. Okuyama et al. (163) found that oral EGF administration to newborn rabbits significantly reduced the spontaneous bacterial translocation that is observed with formula feeding, and could increase the goblet cell number observed in the small intestine. Whether EGF can reduce bacterial colonization of an injured mucosa has not yet been investigated.

Gastric ulcer induction results in markedly elevated levels of bacterial colonization at the ulcer site, which in turn delays ulcer healing (240). Having previously demonstrated that EGF administration could significantly inhibit bacterial colonization of healthy intestinal mucosa (178), experiments were needed to determine if accelerated gastric ulcer healing observed with EGF administration was due to decreases in bacterial colonization. Therefore, the aim of this study
was to examine the effects of oral EGF administration in a model of gastric ulcer with pre-existing bacterial colonization.

### 6.2 Materials and Methods

**Effect of Epidermal Growth Factor Administration.** Previous work using the acetic acid-induced gastric ulcer model in rats has revealed that there is significant bacterial colonization within the ulcer bed when compared to normal stomach (240). In order to determine if daily EGF administration had an effect on the bacterial levels within the ulcer bed, ulcers were induced in rats via serosal application of acetic acid (see Section 2.2). On the seventh day after ulcer induction, a 7-day treatment period was initiated during which recombinant human EGF (1 or 100 µg/kg; Austral Biologicals, San Ramon, CA) was orally administered once-daily. The oral route of administration was chosen since EGF is released into the gut lumen with salivary and duodenal secretions (150). The vehicle for EGF was sterile water and control rats received the same volume of vehicle. For comparison, the effect of twice-daily oral treatment with the combination of streptomycin (336 mg/ml; 0.25 ml) and penicillin (168 mg/ml; 0.25 ml) was also determined. This treatment regimen has previously been shown to be capable of significantly reducing total aerobic bacterial levels and significantly accelerating healing using this ulcer model (240). Fourteen days after ulcer induction, the rats were killed by cervical dislocation, the stomach was removed for ulcer area determination (see Section 2.2), and tissue samples were taken for bacterial culturing (see Section 2.4). In addition, the differences in bacterial levels in the ulcer beds of rats treated with vehicle, EGF or antibiotics were calculated and expressed as a percentage of the average number of bacteria recovered from the vehicle-treated group. This value was referred to as percent clearance. Body weight was measured daily throughout the study.

**Direct Effects of EGF on Bacteria in vitro.** The effects of EGF on bacterial growth were determined in vitro. Three bacterial isolates were used for
these studies: 1) Gram-positive *Enterococcus faecalis*, isolated from fresh rat feces as a single colony grown on tryptic soy broth (TSB; Difco Labs., Detroit, MI) agar plate for 18 hr at 37°C, and 2) Gram-negative *E. coli*, isolated from fresh rat feces as a single colony grown on TSB agar plate for 18 hr at 37°C, and 3) the streptomycin-resistant strain of *E. coli* (C-25) which has previously been shown to delay healing of gastric ulcers in rats (240). Identification of the bacterial isolates from the fresh feces as *Enterococcus faecalis* and *E. coli* were performed at the Veterinary Pathology Laboratory (Alberta Ltd., Edmonton, Alberta, Canada) using standard sensitivity testing assays. All bacterial stock cultures were stored at -70°C in TSB coated onto Microbank™ porous beads (Pro-Labs Diagnostics, Richmond Hill, ON.). In a series of three experiments, log phase bacteria (10^3 CFU/mL) were added in duplicate to wells on a 96 well plate containing TSB with either no EGF (control), or 10 μM EGF, in a total volume of 100 μL/well. This concentration was chosen to reflect the higher end of EGF levels that may be encountered by gastrointestinal bacteria in vivo (241), and is consistent with previous studies using similar experimental protocols of oral EGF administration in infected animals (178). At 1 h intervals (0-5 h post-inoculation) viable bacterial cells in each well were counted by serial dilution and culture on TSB agar plates (for cocci) or MacConkey agar plates (for *E. coli*) for 18 h at 37°C. Bacterial numbers are expressed as log_{10} CFU/mL.

**Effect of EGF on inflammatory cell infiltration.** Myeloperoxidase (MPO) is an enzyme that is found predominantly in the azurophilic granules of neutrophils, and may be used as a quantitative index of inflammation (242). An additional group of rats that had ulcers induced and underwent treatment with either vehicle, EGF (100 μg/kg) or the streptomycin/penicillin combination had tissue samples from the ulcer site taken and processed for the myeloperoxidase assay as previously described (243). Results are expressed as units of myeloperoxidase activity per milligram tissue.

**Transmission Electron Microscopy.** Tissue samples taken from the ulcer bed were fixed in 5% gluteraldehyde in 5% phosphate buffered saline, pH 7.4 at
20°C. Specimens were washed in buffer, postfixed in 1% OsO₄, and dehydrated in increasing concentrations of distilled ethanol. Samples were cleared with propylene oxide, infiltrated and embedded in Spurr's low-viscosity medium (J.B. EM Services Inc., Dorval, PQ). Thin sections (90 nm) were double-stained with saturated uranyl acetate in 50% ethanol, followed by 0.4% lead citrate (244). Specimens were examined on a Hitachi 7000 transmission electron microscope.

**Effects of EGF on acid secretion.** EGF is able to regulate gastric acid secretion, which may in turn affect both the levels of bacteria colonizing the ulcer site and the rate of ulcer healing. To determine the effect of EGF on acid secretion, a continuous gastric perfusion system was used (see Section 2.5). Ulcers were induced in rats by serosal application of acetic acid. On the seventh day after ulcer induction, daily oral administration of EGF (100 μg/kg) began. Basal and pentagastrin-stimulated acid secretion were measured in rats that had received EGF for either 3 or 7 days. Acid output was also measured in rats that had received an equal volume of vehicle (distilled water) for the same amount of time. Each group consisted of 4 or 5 rats.

**Statistical analysis.** All data are expressed as mean ± SEM. Comparisons between groups of data were made using a one-way ANOVA and a Dunnett multiple-comparisons test. Values of $P<0.05$ were considered significant.

### 6.3 Results

**Effects of EGF administration.** Rats treated with vehicle from days 7 to 14 after ulcer induction had a mean ulcer area of $38.1 \pm 6.4 \, \text{mm}^2$ (Figure 6.1). Daily administration of EGF significantly reduced gastric ulcer area. Treatment with EGF at a dose of 1 μg/kg resulted in a mean ulcer area, on day 14, of $16.1 \pm 5.3 \, \text{mm}^2$ ($P<0.05$ compared to vehicle), while EGF at 100 μg/kg resulted in a mean ulcer area of $12.1 \pm 3.1 \, \text{mm}^2$ ($P<0.01$ compared to vehicle). Consistent with previous findings, daily treatment with the streptomycin/penicillin combination significantly
reduced ulcer area, to a mean of 12.6 ± 2.6 mm² (P<0.01 compared to vehicle-treated animals). The reduction in gastric ulcer area observed with both doses of EGF was not significantly different from that observed with antibiotic administration.

Bacterial numbers in gastric ulcer samples following seven day treatment with either vehicle, EGF or streptomycin/penicillin are illustrated in the top panel of Figure 6.1. Rats receiving vehicle over the seven-day treatment period had a mean aerobic bacterial level of 6.5 ± 0.2 log₁₀ CFU/g tissue at the ulcer site, a level significantly (P<0.01) elevated over those seen in samples taken from the stomach of rats without ulcers (~3-4 log₁₀ CFU/g tissue; (240)). Administration of EGF, at both 1 µg/kg and 100 µg/kg, significantly (P<0.01) reduced aerobic bacterial levels (5.0 ± 0.4 and 5.3 ± 0.3 log₁₀ CFU/g tissue, respectively) relative to the rats receiving vehicle. Treatment with the streptomycin/penicillin combination also resulted in a marked reduction in total aerobic bacterial colonization at the ulcer site (4.9 ± 0.3 log₁₀ CFU/g tissue; P<0.01 versus vehicle-treated animals). The greatest bacterial clearance was observed with the streptomycin/penicillin combination with respect to total aerobic bacteria (97.5%). Weight gain over the seven-day treatment period did not significantly differ among any of the groups (data not shown).

Effects of EGF on bacteria in vitro. In order to assess whether the EGF-induced reduction in bacterial colonization observed in vivo was due to a direct anti-bacterial effect of EGF, growth of three bacterial isolates was assessed in vitro in the presence or in the absence of EGF. Endogenous enterococci and E. coli, as well as the laboratory strain, E. coli C-25, have been shown to delay healing of experimental gastric ulcer in rats (240). In medium without EGF, mean bacterial growth between 0 and 5 h for gram negative E. coli, the gram positive Enterococcus faecalis, and E. coli (C-25) was 2.13, 1.31, and 1.84 log₁₀ CFU/ml, respectively. No difference in bacterial proliferation was observed when 10 µM EGF was added to the medium for either isolate (Figures 6.2 and 6.3).

Effects of EGF on inflammatory cell infiltration. Significant inflammatory cell infiltrate was observed in ulcers of each group. Cells involved were
neutrophils, eosinophils and mast cells. A representative micrograph (Figure 6.4) demonstrates the influx of inflammatory cells. Neutrophil infiltration was confirmed using the myeloperoxidase assay. Control gastric tissue taken from rats without ulcers had very low MPO levels (13.1 ± 2.4 U/mg tissue) (Figure 6.5). Ulcer induction resulted in a significant elevation of MPO levels. Vehicle-treated rats had an MPO level of 113.1 ± 15.4 U/mg tissue (P<0.05 versus control rats) and treatment with either the streptomycin/penicillin combination (129.6 ± 28.2 U/mg tissue) or 100 μg/kg EGF (112.4 ± 20.0 U/mg tissue) did not affect MPO concentrations compared to vehicle-treated rats. Twenty-one days after ulcer induction, a time at which the ulcer has previously been shown to be macroscopically healed (240), the MPO had decreased to 47.0 ± 10.7 U/mg tissue, a value not significantly different from control.

*Effects of EGF on acid secretion.* Pentagastrin infusion significantly increased acid output in both vehicle and EGF-treated groups at both the 3-day and 7-day dosing time points, relative to the corresponding basal output (Figure 6.6). No difference in acid secretion was see between the vehicle and EGF-treated rats during any of the different collection periods.
**Figure 6.1.** Effects of daily oral administration of vehicle, a streptomycin/penicillin combination, or recombinant human epidermal growth factor (EGF) on healing of gastric ulcers (bottom) and on the levels of bacteria colonizing the ulcer site (top). Rats were orally dosed from day 7 to day 14 after ulcer induction. Bacterial levels were calculated from serial dilutions on tryptic soy agar plates. The percent clearance, indicated in parentheses over the treatment groups, was calculated from absolute numbers of bacteria recovered per gram of gastric tissue as: (total number of bacteria in a vehicle treated sample – total number of bacteria in a treatment group sample) / total number of bacteria in a vehicle treated sample. **P<0.05, * P<0.01 compared with the group receiving vehicle. Each group consisted of at least 5 rats.
Figure 6.2. Effects of EGF on in vitro proliferation of gram negative *E. coli* (bottom) and gram positive *Enterococcus faecalis* (top). Proliferation of gram positive *Enterococcus faecalis* and gram negative *E. coli* was assessed in the absence (□) or presence (▲) of 10 μM EGF added to the medium. Values are means ± SEM for three experiments run in duplicate. No significant inhibition of proliferation was observed for either gram positive or gram negative bacteria in the presence of EGF when compared to bacteria grown in the absence at any of the time points.
Figure 6.3.  Effect of EGF on the *in vitro* proliferation of *E. coli* (C-25). The *in vitro* proliferation of *E. coli* C-25 was determined in the absence (□) or presence (▲) of 10 μM EGF. Values are means ± SEM from three experiments run in duplicate. The addition of EGF to the culture medium did not significantly affect the proliferation of *E. coli* (C-25) at any of the time points tested.
collagen deposition (→) was seen throughout ulcer tissues in all groups. Bar = 1 μm. Extensive infiltration was observed in ulcers of rats from each treatment group. Infiltrating cells the ulcer bed of a vehicle-treated rat on day 14. Significant inflammation was seen.
Figure 6.5. Myeloperoxidase (MPO) activity of tissue samples taken from the ulcer bed of rats treated with vehicle, an antibiotic combination or recombinant human EGF (100 μg/kg). The test compounds were orally administered from day 7 to day 14 after ulcer induction. Myeloperoxidase activity was significantly elevated in all three groups measured at day 14, compared to control animals that did not have gastric ulcers (naive). * P<0.05, ** P<0.01 compared to control rats that did not have gastric ulcers (naive). Each group consisted of at least 5 rats.
Figure 6.6. Effect of administration of EGF (100 µg/kg) or vehicle on basal and pentagastrin-stimulated acid secretion in the rat. Starting on the seventh day after ulcer induction, rats were orally dosed once daily with EGF or vehicle for 3 or 7 days. Pentagastrin infusion resulted in significantly elevated levels of acid secretion in all groups. EGF administration did not affect acid secretion during any of the collection periods. * P<0.05, ** P<0.01 compared to corresponding basal levels of acid secretion. Each group consisted of 4 or 5 rats.
6.4 Discussion

The epithelium of the gastrointestinal tract is one of the most rapidly proliferating tissues in the body. Epidermal growth factor is intimately involved in the maintenance of mucosal integrity through its ability to stimulate DNA synthesis and subsequently regulate the proliferation and differentiation of a wide variety of cell types in the gastrointestinal tract. Extensive work has detailed these effects of EGF and its ability to prevent and accelerate the healing of gastric ulcers (125,175,203,216,222,229,231,245).

Findings from this study demonstrate that, together with its ulcer healing properties, EGF inhibits bacterial colonization in gastric ulcers. Daily oral administration of recombinant human EGF, at 1 and 100 μg/kg for one week, resulted in a significant reduction in gastric ulcer area. (Figure 6.1). Consistent with previous findings, daily treatment with the streptomycin/penicillin antibiotic combination also significantly reduced gastric ulcer area following a one-week dosing regimen (240), and the degree of healing was similar to that seen in EGF-treated rats.

Endogenous EGF is critical in the healing of chronic ulceration as the removal of the salivary glands in several different models results in a significant delay in wound healing (203,229,232-235). Further, Wright et al. (222) found that ulceration of the human epithelium in the gastrointestinal tract induces the development of a novel cell lineage from stem cells that contains and secretes abundant amounts of immunoreactive EGF. It has been suggested (246) that EGF secretion may occur in response to the presence of an ulcer as there is an observable increase in the EGF concentration in the gastric juice of patients with duodenal ulcers. Working in concert with the increased secretion of EGF when ulceration is present, there is a notable increase in EGF receptor number (125,203,216,245). Following gastric ulcer induction with acetic acid, a 75-fold increase of EGF receptors has been observed immunohistochemically, and this elevated level persisted for up to 16 days following ulcer induction (203) in one
The enhanced gastric ulcer healing observed with exogenous administration of EGF is consistent with previous observations that luminal EGF is capable of stimulating growth and repair when given to the damaged bowel of rats and humans (175, 226, 229, 247).

Recent work using the acetic acid ulcer model, revealed significantly elevated levels of bacterial colonization within the gastric ulcer bed as early as twelve hours after ulcer induction (240). The presence of bacteria in the ulcer bed prolongs the chronicity of the ulcer (115-117, 120, 240, 248). Previous work in which EGF was administered to rabbits (178) prior to oral inoculation with enteropathogenic E. coli demonstrated that EGF inhibits bacterial colonization, which in turn reduces the extent of microvillus injury and disaccharidase deficiencies caused by the infection. The present study sought to determine whether the EGF-induced gastric ulcer healing was due to a reduction of bacterial colonization in the ulcer bed. Consistent with the previous results (178), we observed a significant reduction in bacterial colonization of ulcers following the seven day dosing with EGF at both 1 and 100 μg/kg (Figure 6.1). The reduction in colonization observed was similar to that seen with the administration of the streptomycin/penicillin combination. To assess whether EGF may be exerting a direct bactericidal activity, studies examined the effects of EGF on bacterial growth in vitro. Physiological concentrations of EGF do not affect the growth of either gram-positive Enterococcus faecalis, gram-negative E. coli, nor E. coli C-25, all of which have been previously shown to be capable of colonizing and delaying the healing of gastric ulcers in rats (240) (Figures 6.2 and 6.3). Thus, EGF did not reduce bacterial colonization and accelerate gastric ulcer healing via a bactericidal action. This is consistent with results reported by Okuyama et al. (163) who examined the effect of EGF on bacterial translocation in newborn rabbits. These authors reported that EGF was capable of significantly reducing spontaneous bacterial translocation without significantly affecting small bowel bacterial colonization.
Neutrophil infiltration is the hallmark of inflammatory disorders of the gastrointestinal tract (249). Using transmission electron microscopy, we observed a pronounced neutrophil-predominant inflammatory response in tissues taken from the gastric ulcer bed of vehicle-treated animals (Figure 6.4). This observation was confirmed by high levels of myeloperoxidase activity in ulcer tissue samples (Figure 6.5). As neutrophils are capable of releasing a wide array of substances that can destroy cells and dissolve connective tissue, thereby retarding wound healing, the effect of EGF-treatment on neutrophil infiltration needed to be determined. Results from the MPO assay clearly demonstrated that neutrophil infiltration in ulcer tissues were not different among the EGF- or antibiotic-treated rats, or in the rats that received vehicle alone (Figure 6.5). These results are concordant with the inability of exogenous EGF administration, following the induction of experimental colitis in rats, to reduce the amount of intestinal inflammation as assessed by the MPO assay (225). This suggests that the effects of EGF were not attributable to altered neutrophil infiltration into the damaged tissue.

In addition to its anti-infective properties and proliferative effects on tissues, EGF is capable of regulating gastric acid secretion. Following short-term administration of EGF, inhibition of agonist-stimulated acid secretion has been observed in humans (152), dogs (153) and rats (154) and in vitro in parietal cells or gastric glands from guinea pigs (155), rats (156) and rabbit (151). In vitro findings suggest that EGF exerts a direct effect on the parietal cell to reduce acid secretion (143). In contrast, Chew et al. (151) demonstrated, using rabbit gastric parietal cells, that long-term administration of EGF can stimulate acid secretion. Multiple studies have demonstrated an increase in EGF levels when ulceration is present (125,203,216,222,245). Together with the daily oral administration of EGF during the present study, the concentrations of EGF in gastric tissue may have reached levels sufficient to stimulate, rather than inhibit acid secretion. The primary role of acid in the stomach is to kill bacteria. Indeed, a prolonged reduction in acid secretion predisposes to infection with a variety of bacteria (250,251). In this context, an increase in acid secretion could theoretically aid in ulcer healing.
course, there is substantial evidence from both animal models and human studies to demonstrate that suppression of acid secretion results in accelerated ulcer healing (29,159,252).

In the present study, we found that EGF administration did not influence gastric acid secretion. Treatment with EGF for 3 or 7 days, starting on the seventh day following ulcer induction, did not alter acid secretion compared with rats that had received vehicle over the same time period. Thus, the accelerated ulcer healing was not due to an alteration in acid secretion.

The mechanisms through which EGF inhibits bacterial colonization and promotes ulcer healing require further investigation. EGF is capable of stimulating the synthesis of collagen (175) and this may have also aided in the formation of granulation tissue and the subsequent healing of the mucosal defect. Furthermore, EGF is a very powerful mitogen for fibroblasts (140), which are key elements in the formation of granulation tissue at the base of the ulcer that subsequently aids in the healing process. EGF has been shown to modulate F-actin assembly via activation of small GTP-binding proteins in fibroblasts (238), and may therefore inhibit cytoskeletal alterations associated with cell injury.

EGF is capable of elevating goblet cell number (163) and stimulating mucin glycoprotein synthesis and secretion (164). Decreased goblet cell mucus production following ischemia and reoxygenation of intestinal mucosa has correlated with increased bacterial translocation across intact, viable mucosal epithelium (177), and depletion of mucus secretion results in a marked increase in bacterial passage across an intact ileum in vitro (253). In the inflamed colonic mucosa in rats, a marked increase in EGF-receptor immunoreactivity has been noted and localized to goblet cells (225). EGF is capable of attenuating jejunal mucosal injury induced by oleic acid, in part, through the stimulation and production of mucus (254). Mucus aids in preventing bacterial adhesion to epithelial cells which is a prerequisite for internalization or invasion and it may act as a chemical trap by providing binding sites for bacterial adhesins. Thus, it is possible that EGF may increase host natural defenses by increasing mucus secretion, thereby establishing a dilutional barrier that can prevent bacterial
adhesion and limit injury caused by ingested or administered noxious agents, as well as scavenge toxic oxygen metabolites (255).

Blood flow is critical to ulcer healing. EGF is a potent vasodilator in the gastric mucosal vasculature of the rat (160,161). Blood supplies oxygen, nutrients and peptide hormones, which are essential to maintaining and/or restoring the proper structure, function and turnover of the mucosa. An adequate blood supply also helps sustain the secretion of mucus and bicarbonate, removes hydrogen ions that may back-diffuse, and aids in diluting and removing noxious substances from the interstitium (254). An increased oxygen supply to the ulcer margin is essential to rapid ulcer healing (236). The administration of EGF over the 7-day treatment period in this study may have aided in maintaining blood flow so as to facilitate ulcer healing.

Acting in concert with the ability of EGF to increase blood flow and mucus production is the capacity of EGF to induce cyclooxygenase (COX) and the subsequent production of prostaglandins (168,169). Prostaglandins are capable of stimulating the secretion of mucus and bicarbonate, maintaining mucosal blood flow and enhancing the resistance of epithelial cells (256). In addition, there are data suggesting that prostaglandins may modulate mediator release from inflammatory cells (256). All of these functions are important in gastric ulcer healing and may have contributed to the effects observed in this study.

Having demonstrated that there is a significant reduction in the levels of bacteria colonizing the ulcer site following EGF administration, it is worthwhile to note that this decrease in bacterial numbers could be a consequence of a decrease in ulcer size. EGF may not be acting primarily to reduce bacterial numbers. We have previously shown that the presence of bacteria within the ulcer bed can inhibit gastric ulcer healing (240). The parallel decrease in bacterial colonization and ulcer area observed with antibiotic and EGF administration suggests that EGF may be reducing bacteria numbers, but no direct effect was observed in vitro. EGF possesses a myriad of effects and it is possible that it is through another effect that a reduction in bacteria and accelerated ulcer healing were observed. While we cannot conclusively say that a reduction of bacterial
numbers is the primary mechanism of action of EGF, we have provided evidence to rule out some alternative, potential mechanisms (i.e., acid secretion, neutrophil infiltration).

In summary, the present study demonstrated that oral administration of recombinant human EGF significantly reduced bacterial colonization of gastric ulcers. This effect is not due to a direct anti-bacterial effect of EGF. EGF-treatment did not affect neutrophil infiltration nor acid secretion. Together, these findings show that EGF enhances ulcer healing, at least in part, by inhibiting bacterial colonization in the damaged mucosa via mechanisms that are independent of a bactericidal or anti-inflammatory effect.
CHAPTER 7

EFFECTS OF PGV 20229, A DUAL CYCLOOXYGENASE/LIPOXYGENASE INHIBITOR, ON HEALING OF EXPERIMENTAL GASTRIC ULCERS

7.1 Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used to treat inflammation and pain. However, NSAID use is associated with serious side effects, most notably gastrointestinal (GI) ulcers and bleeding. NSAID-related GI complications may account for the largest number of deaths attributable to any class of therapeutic agent in the United States (257).

NSAIDs inhibit the activity of the enzyme cyclooxygenase (COX), which is responsible for the conversion of arachidonic acid to bioactive prostanoids (3). COX exists in at least two different isoforms. COX-1 is found in virtually all tissues under basal conditions, including the gastrointestinal tract, endothelial cells and platelets (94,100,258,259) and is believed to be responsible for the production of prostaglandins involved in the maintenance of essential physiological functions (e.g. GI mucosal integrity). COX-2, normally undetectable in most tissues, is induced at sites of inflammation in response to mitogens and pro-inflammatory cytokines (191,260) and has been proposed to be responsible for the production of inflammatory prostaglandins. Thus, popular belief follows that because COX-2 is induced, and COX-1 is constitutive, the anti-inflammatory actions of NSAIDs are largely the result of inhibition of COX-2 at sites of inflammation, whereas the adverse effects, such as GI toxicity and bleeding, are primarily due to the inhibition of COX-1. Recent work using selective inhibitors of COX-2 challenges this concept as there is evidence to suggest that COX-2 plays an important role in down-regulating inflammation and accelerating repair, while COX-1-derived prostanoids participate in inflammatory processes.
Studies of compounds that selectively inhibit COX-2 have demonstrated anti-inflammatory activity and reduced ulcerogenic tendencies, but these investigations were restricted to healthy animals and humans (85,99-103). Substantial evidence exists to suggest that COX-2 inhibitors may exhibit adverse effects in the GI tract when given in situations of pre-existing inflammation. In a rat model of colitis, prostaglandin synthesis by the colon was elevated approximately 25-times above that observed in normal colon (21). Administration of several COX-2 selective agents at doses that did not inhibit COX-1 suppressed this colonic prostaglandin synthesis and exacerbated the colitis. In fact, a high rate of perforation and death was observed. Similar observations have been made in gastric tissue. Increased prostaglandin production has been observed in gastric ulcers in mice and this was suggested to be occurring through the induction of COX-2 (104). Marked induction of COX-2 messenger RNA (mRNA) has been demonstrated in gastric mucosal erosions (104,109) and ulcers (104-106), and the disappearance of this COX-2 expression correlated with the healing of the mucosal lesions (104,105). The administration of selective inhibitors of COX-2 activity (NS-398, L745,337) in these instances produced a marked impairment in gastric ulcer healing (104-107), clearly indicating that COX-2 may be beneficial in resolving inflammation.

Is there a clear association between suppression of COX-2 activity and anti-inflammatory effects? Following the suggestion that prostaglandins at sites of inflammation are derived exclusively from COX-2, several studies have provided evidence that in some circumstances, COX-1 produces prostaglandins that contribute to inflammation. Carrageenan-induced inflammation in the rat paw has been shown to be significantly reduced with the administration of an NSAID that is more selective for COX-1 (aspirin) (261) and several selective inhibitors of COX-2 activity significantly reduce inflammation in this model only when given at doses that inhibit COX-1 (262). Furthermore, in human bursitis a selective COX-2 inhibitor suppressed prostanoid production only at concentrations that also inhibited COX-1 (263), further supporting a role for COX-1 in inflammation.
5-lipoxygenase (5-LO) is another major enzyme involved in the metabolism of arachidonic acid. 5-lipoxygenase is responsible for the conversion of arachidonic acid to leukotrienes (LT) as well as other biologically active 5-lipoxygenase metabolites (5-HPETE and 5-HETE). Leukotrienes have potent phlogistic activity (261). LTB₄ is a potent chemotactic factor for neutrophils and the peptidyl leukotrienes (LTC₄, LTD₄, LTE₄) cause increased vascular permeability (264). Inhibition of leukotriene synthesis has been shown to reduce the severity of NSAID-induced gastric injury (265,266). Cells involved in the inflammatory process release metabolites of arachidonic acid which may exacerbate an inflammatory condition. Therefore, inhibition of 5-lipoxygenase may decrease the number of cells present to produce inflammatory mediators thereby reducing the magnitude of an inflammatory response.

Given the potential contributions from both cyclooxygenase and lipoxygenase to inflammatory responses, dual inhibitors of lipoxygenase and cyclooxygenase may be beneficial and exhibit anti-inflammatory activity with a broader spectrum than that of classical NSAIDs. Furthermore, it may be beneficial for a compound to have both cyclooxygenase and lipoxygenase inhibitory activities since prostaglandins have been suggested to enhance LTB₄-mediated inflammation (23).

In the present study we examined the effects of PGV 20229 (a COX-1/COX-2/5-LO inhibitor), celecoxib, (a highly selective COX-2 inhibitor; >300 fold selectivity for COX-2 over COX-1 in vitro; (103)) and zileuton, (a 5-LO inhibitor) on gastric ulcer healing.

### 7.2 Materials and Methods

**Effects of test drug administration on gastric ulcer healing.** Gastric ulcers were induced in rats through the serosal application of 80% acetic acid (see Section 2.2). On the third day after ulcer induction, the rats were weighed and
were then given one of the following drugs orally: celecoxib (5, 15 or 45 mg/kg),
zileuton (5, 15 or 45 mg/kg) or PGV 20229 (15, 45 or 125 mg/kg). Control rats
were treated with the same volume (1mL/kg) of vehicle (1% carboxymethylcellulose). The drugs were administered every 12 hours from day 3
to day 10 post-ulcer induction. Two hours following the final dose, the rats were
killed. The stomach was removed and pinned out for ulcer area determination (see
Section 2.2). Gastric tissue samples were taken from at least five rats per
treatment group for gastric eicosanoid determination (see Section 2.3). In addition,
a blood sample was drawn from the descending aorta for determination of
hematocrit, thromboxane, leukotriene and prostaglandin production. Body weight
was measured daily throughout the study.

**Effects of test drug administration on whole blood thromboxane synthesis.** In platelets, thromboxane B₂ is generated almost exclusively via the
COX-1 pathway. Thus, the effects of test drug administration on COX-1 activity
can be measured by taking a whole blood sample, allowing it to clot for 45 min at
37°C, and then measuring thromboxane B₂ concentrations using an ELISA (see
Section 2.3). This also provides an index of bioavailability of the drug.

**Effects of test drug administration on gastric eicosanoid synthesis.**
Gastric tissue samples were taken from the side of the stomach contralateral to the
gastric ulcer in order to determine any effects of test drug administration on gastric
prostaglandin E₂ (PGE₂) and leukotriene B₄ (LTB₄) synthesis. Prostaglandin E₂
and leukotriene B₄ concentrations were determined using ELISAs (see Section
2.3).

**Effects of test drug administration on cyclooxygenase-2 activity.** In
order to determine the COX-2 inhibitory activity of the various test drugs, we
utilized the carrageenan airpouch model in the rat. Inflammatory prostaglandins
recovered from the airpouch have previously been reported to be derived primarily
from COX-2 (85,267). An airpouch was induced in the intrascapular area of the
back of a rat, as previously described (85). Briefly, rats were lightly anesthetized
(5% halothane; v/v) and 20 mL of air were injected subcutaneously into the back of
the rat on day one. An additional 10 mL was injected in the same area on both day
three and day six. On day six, the rats were fasted overnight with free access to drinking water. Following the overnight fast, the rats were divided into treatment groups and were orally dosed (1 mL/kg) with either: celecoxib (1, 5, 15, or 45 mg/kg; N=5 rats/group), zileuton (5, 15, or 45 mg/kg; N=5 rats/group) or PGV 20229 (15, 45, or 125 mg/kg; N=4 rats/group) one hour prior to the installation of carrageenan. Control rats received the vehicle (1% carboxymethylcellulose). Carrageenan was prepared as a 1% solution (w/v) in sterile saline, and 2 mL were injected into the airpouch under light anesthesia with 5% halothane (v/v). The animals were left for six hours with no access to food or water. The rats were then anesthetized with pentobarbital (65 mg/kg) and 1 mL of heparinized saline was injected into the airpouch. A small incision was made in the pouch. The exudate was recovered and transferred to a sterile tube. An aliquot of the exudate was used to determine leukocyte infiltration using a Coulter counter (model Z1). An additional aliquot was frozen on dry ice and stored at -20°C for PGE2 and/or LTB4 determination using commercially available ELISA kits (see Section 2.3). The stomach was removed and examined.

**Gastric cyclooxygenase-2 mRNA expression.** Gastric COX-2 expression were examined using the reverse-transcription polymerase chain reaction (RT-PCR). Samples of the corpus region of the stomach along the ulcer margin were taken from rats that did not receive any of the test drugs. The samples were immediately frozen on dry ice in a 50% (w/v) guanidinium solution (containing: 26.4 mmol/L sodium citrate (pH 7.0), 0.528% sarcosyl, and 0.0072% β-mercaptoethanol). 1 ml of guanidinium solution was used per 100 mg of tissue. Total RNA was isolated using the acid guanidinium isothiocyanate method, as previously described (268).

Briefly, 1 μg of RNA from each sample was reverse transcribed at 42°C using Superscript RNAse H Reverse Transcriptase (Gibco BRL, Gaithersburg, MD) and the reaction mixture (containing: 2 μL of 10x PCR Buffer, 2 μL of 10 mmol/L deoxynucleoside triphosphate (dNTP) stock, and 2 μL of N6 random hexamer stock). The enzyme was deactivated by heating the samples to 95°C for 10
minutes. Following the reaction, 2 μL of complementary DNA (cDNA) was mixed with 5 μL of 10x PCR Buffer. The upstream primer (2 μL; 10 pmol/μl primer) and the downstream primer (2 μL; 10 pmol/μl primer) to be examined were then added to each tube. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

DNA amplification occurred by denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 45 seconds. To ensure complete denaturation of the DNA with no background polymerase activity, Taq DNA polymerase was added to the PCR mixture at the hot start of cycle 1. The COX-2/GAPDH genes were coamplified for 30/22 cycles, respectively. Therefore, the GAPDH upstream and downstream primers were added to the PCR reaction mixture at the hot start of cycle 13. The optimal number of amplification cycles for COX-2 had previously been determined by a cycle test.

The PCR products were then separated on a 1.5% agarose gel, containing 10 μg of ethidium bromide. The gel was visualized under UV light and quantities of each product were normalized to control levels of the GAPDH gene using a densitometer and the Quantity One software (Bio Rad, Hercules, CA). Results are expressed as densitometry units. The COX-2 primers were based on a previously published sequence for rat COX-2 (269) and the amplicon length of the COX-2 primer (base pairs) was 1815. The upstream primer read as follows: 5'-CAC AGT ACA CTA CAT CCT GAC C-3' and the downstream: 5'-TCC TCG CTT CTG ATC TGT CTT G-3'.

**Statistical analysis.** All data are expressed as mean ± SEM. Comparisons between groups of data were made using a one-way analysis and a Dunnett multiple-comparisons test. For comparisons of COX-2 mRNA levels, a one-way ANOVA and Newman-Keuls test was used. With all analyses, P < 0.05 was considered significant.

**Materials.** Celecoxib, zileuton and PGV 20229 were kindly provided by Dr. John Janusz of Procter & Gamble Pharmaceuticals (Cincinnati, OH). All other
chemicals and reagents were obtained from Gibco BRL (Gaithersburg, MD), Sigma Chemical Co. (St. Louis, MO) or VWR Scientific (Edmonton, AB, Canada).

7.3 Results

**Gastric ulcer healing.** Twice-daily administration of vehicle from days 3-10 after ulcer induction resulted in a mean gastric ulcer area of 45.6 ± 3.5 mm$^2$ (Figure 7.1). PGV 20229, at doses of 15, 45 and 125 mg/kg, did not significantly impair gastric ulcer healing compared to vehicle-treated animals, with mean ulcer areas of 42.6 ± 3.5 mm$^2$, 53.4 ± 4.4 mm$^2$, and 57.3 ± 4.2 mm$^2$, respectively. Likewise, zileuton at 15, 45 and 125 mg/kg had no significant effect on ulcer area (45.1 ± 3.9 mm$^2$, 40.0 ± 2.8 mm$^2$, and 39.1 ± 3.6 mm$^2$, respectively). On the other hand, treatment with celecoxib at 5 mg/kg and 45 mg/kg significantly impaired ulcer healing when compared to controls (mean ulcer area 62.8 ± 3.9 mm$^2$ and 66.8 ± 5.6 mm$^2$; respectively). No significant change in hematocrit was observed in any of the treatment groups when compared to the vehicle-treated group (data not shown).

**Thromboxane synthesis in whole blood.** Whole blood thromboxane B$_2$ synthesis was measured in order to provide an index of both the bioavailability of the compounds and their influence on COX-1 activity. Blood from rats receiving vehicle over the 7-day treatment period produced a mean thromboxane B$_2$ concentration of 6918 ± 516 ng/ml (N=10) (Figure 7.2). None of the three doses of celecoxib tested significantly affected thromboxane synthesis. However, PGV 20229 significantly reduced whole blood thromboxane B$_2$ synthesis at all doses tested. Thromboxane synthesis was reduced by ~93% in rats receiving PGV 20229 at 45 mg/kg and 125 mg/kg, versus vehicle-treated rats. Zileuton also significantly reduced whole blood thromboxane synthesis at the 5 mg/kg, 15 mg/kg and 45 mg/kg doses (~53%, 66%, and 62% reduction, respectively, versus vehicle-treated rats) (Figure 7.2).
**Gastric eicosanoid synthesis.** Figure 7.3 shows the effects of twice-daily administration for seven days of PGV 20229, celecoxib and zileuton on prostaglandin E2 synthesis by the stomach. PGV 20229 significantly suppressed prostaglandin synthesis, approximately 58% at 15 mg/kg, 65% at 45 mg/kg and 73% at 125 mg/kg, relative to that in vehicle-treated rats. Celecoxib, at both 5 and 45 mg/kg, also significantly reduced prostaglandin E2 concentrations ($P<0.01$ versus vehicle-treated rats). Zileuton was without effect on gastric prostaglandin E2 synthesis, at all doses tested.

Gastric leukotriene B4 synthesis following one-week treatment with PGV 20229, celecoxib or zileuton is also shown in Figure 7.3. Neither PGV 20229 nor celecoxib, at any of the doses tested, had a significant effect on LTB4 synthesis. In contrast, zileuton significantly reduced LTB4 synthesis at all three doses.

**Airpouch eicosanoid synthesis.** PGV 20229 significantly reduced exudate PGE2 concentrations at the two higher doses tested, with near-complete suppression at 125 mg/kg (Figure 7.4). All four doses of celecoxib also significantly reduced exudate PGE2 concentrations. Zileuton did not reduce exudate PGE2 levels; rather it significantly elevated exudate PGE2 concentrations at the 5 mg/kg dose. Zileuton significantly reduced exudate leukotriene B4 levels at all doses used (Table 7.1). No macroscopic gastric damage was observed in any of the treatment groups.

**Leukocyte infiltration into the airpouch.** PGV 20229 and celecoxib significantly reduced the numbers of leukocytes in the airpouch exudate at all doses tested for each compound, compared to vehicle-treated rats (Figure 7.4). Both compounds reduced leukocyte infiltration by at least 50% at all doses used. Zileuton did not significantly affect leukocyte infiltration into the airpouch at any dose tested.

**Exudate volume.** The volume of exudate retrieved from the airpouch was 1.9 ± 0.1 ml in vehicle-treated rats. None of the test drugs, at any of the doses used, had a significant effect on the exudate volume.
Gastric COX-2 mRNA expression. COX-1 is the predominant isoform found in the gastrointestinal tract under basal conditions (94). COX-2 is expressed at low levels in most cells, including the human stomach and intestine (94,95). COX-2 mRNA expression has been found in gastric tissues taken from ulcer margins in both mice and rats (104,105). COX-1 mRNA has also been found in ulcer margin tissue, and these levels were similar to that in the control stomachs with no ulcers (105). In addition, these levels did not change during the period of ulcer healing (105). The expression of COX-2 mRNA in gastric tissue was confirmed in the present study using RT-PCR. COX-2 was not detected in gastric tissue taken from naïve rats (no ulcer). This is consistent with previous results in the rat. The gastric mucosa of normal rat stomach was shown to express COX-1 mRNA, while COX-2 was not detected (105). In gastric tissue taken from the ulcer margin, there was a pronounced induction of COX-2 mRNA one day following ulcer induction (6868 ± 176.2 arbitrary densitometry units; normalized to GAPDH). On the third day after ulcer induction COX-2 mRNA expression had decreased to 6266 ± 172.9 (P<0.05 compared to day 1 values). The COX-2 mRNA levels continued to decrease over the course of the next seven days, and on the tenth day after ulcer induction COX-2 mRNA expression was significantly reduced compared to day 1 levels (5987 ± 123.5; P<0.01 compared to day 1 values).
Figure 7.1. Gastric ulcer area following seven-day treatment with various test drugs. PGV 20229, celecoxib or zileuton were orally administered twice-daily from day 3 to day 10 following ulcer induction. Each group consisted of at least 28 rats. *P<0.05, **P<0.01 compared to vehicle-treated group.
Figure 7.2. Effects of various test drugs on whole blood thromboxane synthesis. Whole blood thromboxane synthesis was measured as an index of COX-1 activity. Drugs were orally administered, twice-daily, for one week. Each bar represents the mean ± SEM of 10 rats. **P<0.01 compared to vehicle-treated group.
Figure 7.3. Effects of test drug administration on gastric prostaglandin E₂ (top) and gastric leukotriene B₄ synthesis (bottom). The test drugs were orally administered, twice-daily, for one week. Each bar represents the mean ± SEM of 6 rats. *P<0.05, **P<0.01 compared to vehicle-treated groups.
Figure 7.4. Effects of various test drugs on leukocyte infiltration (bottom) into the airpouch and prostaglandin E₂ levels (top) in the exudate. Each group consisted of 4-5 rats. **P<0.01 compared to vehicle-treated group. *P<0.05 compared to vehicle-treated group.
Table 7.1. Effects of zileuton on airpouch exudate leukotriene B₄ concentrations. Zileuton was orally administered one hour prior to carrageenan instillation in the rat dorsal airpouch. Each group consisted of 5 rats. **P<0.01 compare to the vehicle-treated group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LTB₄ synthesis (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>1517.0 ± 37.9</td>
</tr>
<tr>
<td>Zileuton (5)</td>
<td>927.2 ± 62.4**</td>
</tr>
<tr>
<td>Zileuton (15)</td>
<td>626.7 ± 46.8**</td>
</tr>
<tr>
<td>Zileuton (45)</td>
<td>837.8 ± 110.1**</td>
</tr>
</tbody>
</table>
7.4 Discussion

The demonstration of a second isofonn of cyclooxygenase (COX-2) that is induced during acute inflammation (85,100) or experimentally in response to mitogenic stimuli or certain cytokines (191,260) led to the suggestion that prostaglandins derived from this isofonn were responsible for driving inflammation. In contrast, prostaglandins derived from COX-1, the predominant isofonn found in most tissues, perform “housekeeping” functions by synthesizing prostaglandins that regulate normal cell activity (270). This theory provided an impetus for the development of new NSAIDs that would selectively inhibit COX-2. Early reports demonstrated that these COX-2 selective compounds were anti-inflammatory and exhibited reduced GI toxicity in healthy animals and humans (85,99-102), but in circumstances of pre-existing inflammation, they markedly impaired healing (21,104-106). Furthermore, studies demonstrating a critical role for COX-1 in inflammation (262,263,271) indicated that combined COX-1 and COX-2 blockade may produce anti-inflammatory effects superior to those achieved by COX-2 inhibition alone.

The results of the present study show that PGV 20229, a COX-1/COX-2/5-LO inhibitor, has similar anti-inflammatory actions to celecoxib, a selective COX-2 inhibitor. Unlike celecoxib, however, PGV 20229 does not impair gastric ulcer healing. Zileuton, a 5-LO inhibitor, also had no effect on gastric ulcer healing.

Determination of thromboxane production by blood revealed that PGV 20229 and zileuton significantly suppressed COX-1 activity. Celecoxib did not exhibit any inhibitory activity against thromboxane production, confirming that the doses used in this study spared COX-1.

PGV 20229 administration resulted in a significant reduction in gastric prostaglandin E₂ (PGE₂) synthesis in samples taken from non-ulcerated gastric tissue, at all doses tested. Despite this, gastric ulcer healing was not impaired. Inhibition of PGE₂ synthesis was also observed with celecoxib. Interestingly, inhibition of PGE₂ synthesis was observed at the same two doses of celecoxib that impaired gastric ulcer healing. In healthy rats celecoxib was found to be devoid of
any inhibitory activity on gastric PG synthesis (272). However, in inflammatory conditions it has been noted that there is rapid induction of COX-2 (104,109) and significantly elevated levels of PG production (21,104-106). These increased levels of PGE_2 have been ascribed to COX-2 activity (104,105). The findings of the present study suggest that celecoxib may be acting on COX-2 in the non-ulcerated tissue of the stomach. COX-2 is expressed at low levels in most cells of the gastrointestinal tract, including human stomach and intestine (134). However, it has previously been shown that, despite the presence of the gastric ulcer, COX-2 mRNA expression is not increased in the non-ulcerated tissue of stomachs with ulcers (105). Further, PGE_2 production in the non-ulcerated tissue was not affected by the presence of ulcers in the stomach and that the level was nearly the same as that observed in normal tissue (105,106).

The impaired ulcer healing observed in celecoxib-treated rats is consistent with previous studies of selective COX-2 inhibitors and ulcer healing. Mizuno et al. (104) demonstrated that administration of NS-398, a selective COX-2 inhibitor, significantly retarded ulcer healing in the mouse. This was confirmed in the rat by two independent groups (105,113). Schmassmann et al. (107) reported that L745,337, another selective COX-2 inhibitor, also delayed gastric ulcer healing using a different model in the rat. In addition, nimesulide has been shown to significantly increase the severity of stress-induced gastric ulcers in rats (108). Clinically, the results from the present study may be very important. Serious NSAID-associated complications frequently occur without warning symptoms (257) and a potential danger exists with the widespread use of an agent that might exacerbate or delay the healing of ulcers, thereby increasing the risk of serious ulcer complications.

Zileuton was without an inhibitory activity on gastric ulcer healing, but it did inhibit COX-1, as indicated by the reduced blood thromboxane concentrations. Zileuton inhibits the 5-lipoxygenase enzyme and the subsequent production of leukotrienes (i.e. LTB_4, LTC_4, LTD_4, and LTE_4). LTB_4 stimulates neutrophil chemotaxis (273,274), enhances neutrophil-endothelial interactions (108,274), and stimulates neutrophil activation, resulting in degranulation and the release of
enzymes, reactive oxygen metabolites (22,275) and cytokines (i.e. IL-1 and TNFα) which may contribute to tissue injury (276). The peptidoleukotrienes (LTC₄, LTD₄, LTE₄) are potent vasoconstrictors in the gastric microcirculation (277). Significant reductions in gastric LTB₄ concentrations were observed with zileuton at all doses tested. Thus, zileuton administration may have prevented neutrophil-mediated tissue damage and peptidoleukotriene-induced decreases in blood flow. Gastric blood flow is critical to ulcer healing. NSAID administration has been shown to result in attenuated gastric blood flow at the ulcer margin which corresponded to impaired ulcer healing (113,278). Thus, the inhibition of leukotriene synthesis and the subsequent maintenance of blood flow to the ulcer site may explain the lack of effect of zileuton on ulcer healing.

Administration of PGV 20229 did not impair gastric ulcer healing. This lack of effect on gastric ulcer healing with a dual cyclooxygenase/lipoxygenase inhibitor is consistent with results obtained by Lesch et al. (279). These authors reported that administration of a COX-2/5-LO inhibitor to rats did not impair gastric ulcer healing. Further, they suggested that inhibition of COX-2 alone may impair ulcer healing when 5-LO activity remains undisturbed (i.e., concurrent 5-LO/COX inhibition doesn’t impair ulcer healing). To this end, we have demonstrated, using zileuton, that 5-LO inhibition on its own does not influence ulcer healing.

In order to confirm in vivo effects on eicosanoid synthesis of PGV 20229, celecoxib and zileuton, we employed the carrageenan airpouch model in the rat. The carrageenan airpouch model is characterized by an influx of neutrophils and the increased production of mediators in the exudate such as prostaglandins and leukotrienes (280). The inhibition of leukocyte infiltration into the airpouch may be used as an index of anti-inflammatory activity. Further, greater than 90% of the prostaglandins recovered from the airpouch are derived from COX-2 (85). Therefore, the effects of the various test drugs on prostaglandin E₂ production in the airpouch provides an index of COX-2 inhibitory activity. PGV 20229 reduced leukocyte infiltration into the airpouch as effectively as celecoxib, at all doses used (Figure 7.4). PGV 20229 inhibited prostaglandin E₂ production at the two higher
doses while all four doses of celecoxib reduced the PGE$_2$ levels. Zileuton did not reduce the number of leukocytes infiltrating into the airpouch nor the prostaglandin E$_2$ levels. In fact, at the lowest dose (5 mg/kg) zileuton significantly increased the PGE$_2$ levels. Zileuton administration markedly reduced airpouch exudate LTB$_4$ concentrations, confirming its ability to suppress 5-LO activity (Table 7.1).

The development of selective inhibitors of COX-2 is based on the belief that prostaglandins produced at sites of inflammation are derived solely from that isoform of COX. The observation that arachidonic acid-induced ear swelling in COX-1 deficient mice was markedly reduced when compared to wild-type mice (281), initially suggested a role for COX-1 in inflammation. In a recent study using the rat paw edema assay, several selective inhibitors of COX-2 activity (NS-398, nimesulide, DuP697, etodolac) were found to significantly reduce inflammation only when given at doses that inhibited COX-1 (262). Using the same model, administration of a highly selective COX-1 inhibitor, SC-560, was shown to be capable of reducing inflammatory prostaglandins in the footpad to basal levels (267). In addition, L745,337, a selective COX-2 inhibitor, inhibited prostaglandin concentrations in human bursitis tissue only at concentrations that inhibited COX-1 (263). These results suggest that, in some circumstances, suppression of COX-1 is required to produce anti-inflammatory effects.

The present study demonstrates that administration of PGV 20229, a COX-1/COX-2/5-LO inhibitor, is anti-inflammatory yet spares gastric ulcer healing. NSAIDs are the mainstay of treatment of patients with rheumatoid arthritis and osteoarthritis (99). The delaying effect of the NSAID on ulcer healing and the possibility of serious NSAID-associated complications often require the patient to cease taking these drugs. Drugs such as PGV 20229, that suppress inflammation but do not inhibit gastric ulcer healing, may represent attractive alternatives when cessation of NSAID therapy in arthritic patients is not a viable option.
CHAPTER 8

GENERAL DISCUSSION

The research presented in this dissertation was undertaken to gain insight into the effects of a new class of NSAIDs, bacteria and EGF on gastric ulcer healing. Gastric ulcer healing is a multi-factorial process and by no means is this dissertation to be considered a comprehensive review of all aspects involved in this process. However, a broad subject base was covered. Some studies focused on the role of cyclooxygenase and the quest for the development of an agent that would be anti-inflammatory, yet spare the gastrointestinal tract (see Chapters 4 and 7). Other experiments examined the role of bacteria, outside of the *Helicobacter* species, that were capable of having profound effects on ulcer healing (see Chapter 5). A third set of studies were aimed at examining the effects of a growth factor that has long been recognized to influence ulcer healing, on bacterial colonization and subsequent ulcer healing (see Chapter 6). Detailed analyses and discussions have been presented in the appropriate chapters. Therefore, this chapter will address some additional factors that may have contributed to ulcer healing in the studies described, but have not been considered in the relevant chapters. This chapter will conclude with potential future avenues of research.

The gastric ulcers produced by acetic acid in rats are macroscopically, histologically and ultrastructurally similar to gastric ulcers in humans (182). However, it must be recognized that these ulcers are not completely representative of the human counterpart. Clinically significant ulcers in man occur primarily in the antrum, whereas using this model, ulcers were induced in the corpus region. The area available for acetic acid application is very limited in the antral region of the stomach of fasted rats. Therefore, ulcer induction was performed in the corpus region to produce ulcers that would be appropriately large so as to be capable of observing significant impairment/acceleration of ulcer healing.

In Chapter 5, the role of bacterial colonization in experimental ulcer healing was examined. The clinical relevance of this study could be criticized on the basis
of the possibility that the creation of an ulcer resulted in an environment conducive to colonization, and that this may not be indicative of what happens clinically. Some would argue that colonization of the stomach by *Helicobacter pylori* precedes the development of mucosal injury. However, it is important to consider that *Helicobacter pylori* infection is found in approximately half of the world's population, but only a minority (15%) of the infected population develops peptic ulcer disease (282). Virulence factors have been ascribed to some strains of *H. pylori* that may aid in promoting ulceration. Alternatively, it has been suggested that there must first be a break in the mucosa or a loss of mucosal integrity for *H. pylori* to result in peptic ulcer disease. This may be more consequential than originally recognized. Ulcers have often been observed to occur in the presence of non-toxigenic strains of *H. pylori* (283). In fact, 30-55% of patients with peptic ulcers have been shown to have non-toxigenic *H. pylori* isolates (283). It has been suggested that NSAID ingestion or multiple infection (i.e., infection with a toxigenic and a non-toxigenic strain) may be contributory factors (283). In experimental models, bacteria have been shown to be important in exacerbating mucosal injury in the GI tract caused by NSAID administration (76,131,132). Given the widespread use of NSAIDs (7), it is possible that gastric ulceration in a certain population of NSAID-users is maintained by bacterial colonization. Further evidence for the involvement of bacterial species, other than *Helicobacter*, in gastric ulcer healing comes from studies dating back more than a century; a paper published in 1874 reported *Streptococci* colonizing gastric ulcers (115). Even a number of present day studies have reported *Streptococcal* and *Candidal* colonization of ulcers (115,117,120,127,128). Thus, while most research related to bacterial colonization of the stomach has focused on the role of *H. pylori*, it should be recognized that other species may make a significant contribution to the natural history of ulcers.

The research described in Chapter 6 examined the effects of EGF on gastric ulcer healing and bacterial colonization. We observed a significant reduction in ulcer size and a concomitant decrease in bacterial colonization at the ulcer site. However, EGF did not have a bactericidal action *in vitro*. As alluded to in the
chapter, other mechanisms may have been involved in accelerating ulcer healing, subsequently resulting in a reduction in ulcer area available for colonization. In addition to the other effects of EGF that may have contributed to ulcer healing (discussed in Section 6.4) some of the potential mechanisms are discussed in the following sections.

It is important to remember that most members of the EGF family can bind to the EGFR to elicit similar cellular responses. The physiological ligand acting at a specific receptor cannot be predicted a priori (141). Thus, cellular activities observed following the addition of one member of the family in vitro may actually be effected by a different member in vivo. The potential arises that transforming growth factor alpha (TGFα), a member of the EGF-related family, may have played a role in ulcer repair. TGFα is normally found in the gastric mucosa in amounts 20-times greater than EGF, but it, like EGF, has been found to be over-expressed in the presence of a gastric ulcer (125). Binding sites in the gastrointestinal tract appear to be equivalent in distribution for EGF and TGFα when either are used as a ligand following the assumption that they share a common receptor (284). Thus, some of the effects observed following EGF administration in our ulcer model may be have been attributable to TGFα.

Ulceration also induces the rapid expression of trefoil peptides (235). The trefoil peptides, rat spasmylytic polypeptide (rSP) and rat intestinal trefoil peptide (rITF), are expressed by the ulcer-associated cell lineage (UACL) in the area of ulceration (285). As trefoil peptides have well documented proliferative effects, the local increase in tissue levels, together with mucosal growth factor expression, may have synergistically stimulated mucosal cell proliferation and ulcer healing (221,235,285). Combinations of EGF plus intestinal trefoil factor have been shown to have synergistic effects on epithelial cell migration and healing responses (286). Furthermore, the expression of the trefoil peptide pS2 at sites of mucosal damage may be stimulated by luminal EGF, as the pS2 gene has an EGF responsive element (287). Thus, it is conceivable that there was increased trefoil peptide
expression in our ulcer model and this increase could have contributed to the observed healing.

Many other growth factors have been shown to be critical in the healing of damaged mucosa, including transforming growth factor β (TGFβ) and basic fibroblast growth factor (bFGF). TGFβ promotes the healing of damaged mucosa by stimulating cell migration and increasing production of the extracellular matrix (288). bFGF is the major growth factor responsible for angiogenesis, and may be found in endothelial cells, fibroblasts and macrophages during the late stages of ulcer healing (6). bFGF is stored within the basement membrane and is released after injury to stimulate tissue repair and healing (166). bFGF has previously been shown to markedly accelerate ulcer healing in the rat (289), possibly by increasing cNOS expression, angiogenesis and reinnervation with sensory nerves.

Additional factors that are involved in ulcer healing but were not specifically addressed in any of the chapters are the polyamines. The polyamines, spermidine and spermine, and their precursor, putrescine are synthesized and stored within eukaryotic cells, such as the cells lining the lumen of the gastrointestinal tract (288). The GI tract is also exposed to polyamines from a variety of other sources: bacteria synthesize and secrete polyamines into the GI lumen, the normal diet contains relatively large amounts of these compounds and polyamines are released from sloughed epithelial cells when they are digested (288). Polyamines are required for normal cell growth and differentiation (290). The first and rate-limiting step in the synthesis of polyamines is the production of putrescine from ornithine. This reaction is catalyzed by the enzyme ornithine decarboxylase (ODC) (288). ODC is present at low levels in inactive cells, but its activity is rapidly increased in the presence of tissue injury or the stimulatory effects of trophic substances, such as growth factors and hormones (288). Binding of EGF or TGFα to their receptor results in an increase in ODC activity (12,141,226). Putrescine is converted to spermidine by the addition of a propylamine group, and spermidine is then converted to spermine by the subsequent addition of another propylamine (288). Polyamines are capable of binding to DNA and affecting the transcription of
genes, such as protooncogenes and those involved in regulation of the cell cycle. Polyamines alter protein synthesis, the activity of enzymes and they can bind to cell membranes resulting in a stabilizing effect (288). In addition, recent experiments have suggested that EGF receptor function is polyamine-dependent (291). Inhibition of ODC activity impairs the healing process (292), while the administration of exogenous polyamines can significantly accelerate the healing of gastric mucosal lesions (293). Thus, polyamines make a significant contribution to repair of damaged GI mucosa.

The studies presented in Chapters 4 and 7 examined the influence of COX-2 on ulcer healing. Contrary to original assumptions (85), the induction of COX-2 and the prostaglandins derived from this isoform are not responsible solely for driving the inflammatory response. We have confirmed that COX-2 is essential to ulcer healing and that its inhibition impairs healing. The critical role of COX-2 in down-regulating inflammation should not be surprising. Prostaglandins have well characterized anti-inflammatory properties (see General Introduction). Mice with a disrupted COX-2 gene had normal acute inflammatory responses but they developed severe renal pathology that led to early death (294). Moreover, the COX-2 knock-out mice have reduced reproductive capabilities and exhibit signs of cardiac fibrosis (295). A subsequent study using mice with a disrupted COX-2 gene has demonstrated that these mice are capable of exhibiting inflammatory responses of a similar magnitude as those observed in the wild-type controls (262). Furthermore, resolution of inflammation was impaired in the mice with the disrupted COX-2 gene (262). Evidence obtained with transgenic animals should be interpreted with some degree of caution, but these findings tend to indicate that COX-2 is vital to survival and important in down-regulating inflammation. Additional evidence in support of this hypothesis may be found in the cardiovascular system. Active COX-2 protein is readily expressed in the smooth muscle component of human blood vessels (258). Mitchell and Warner (258) have demonstrated that in the presence of vascular endothelial damage, COX-2 induction may compensate for the loss of COX-1 and suppress inflammatory events, such as cell proliferation, cytokine release and adhesion receptor
expression, all of which contribute to the pathology of vascular occlusive diseases (258). Thus, contrary to the original theory that COX-1-derived prostaglandins are all “good” and COX-2-derived prostaglandins are all “bad”, it appears that COX-2 expression may be beneficial.

**Future Directions for GI-Sparing Anti-inflammatory Drugs**

A large amount of time and resources have been invested in the development of selective COX-2 inhibitors. As demonstrated in Chapter 7, chronic administration of a selective COX-2 inhibitor resulted in impaired gastric ulcer healing, which was consistent with previous studies. A need exists for an anti-inflammatory agent that will spare the GI tract, and not impede healing or exacerbate pre-existing GI inflammation. The NO-NSAIDs would appear to fit this description, since they exhibit anti-inflammatory, analgesic, anti-pyretic and anti-thrombotic effects, but are also capable of accelerating the healing of gastric ulcers. As a logical extension of the ability of an NO-NSAID to accelerate ulcer healing in an experimental model, it will be of interest to see if NO-NSAIDs are capable of similar actions in the clinical setting.

The recent report from Gilroy et al. (296) further supports the contention that COX-2-derived prostaglandins exert anti-inflammatory properties. In carrageenan-induced pleurisy in the rat, two peaks of COX-2 protein expression were observed. The first peak occurred 2 hours following carrageenan-injection, and this was associated with neutrophil infiltration and maximal PGE₂ production (296). At 48 hours there was second peak, with a 350% increase in COX-2 protein expression compared with the levels at 2 hours. Monocytes/macrophages were the predominant infiltrating cells at this time. This second peak coincided with resolution of inflammation and was associated with low levels of PGE₂ synthesis but markedly elevated levels of prostaglandin D₂ and 15deoxyΔ12-14 prostaglandin J₂ (296). The authors postulated that this mononuclear-derived, second peak in
COX-2 expression may aid in the resolution of inflammation (296). Their reasoning was as follows. In stimulated macrophages, the nuclear receptor peroxisome proliferator-activated receptor-gamma (PPAR-γ) is markedly up-regulated (297). In these activated macrophages, prostaglandins of the J series (prostaglandin D₂ metabolites) can bind to and activate PPAR-γ, resulting in various anti-inflammatory events. Thus, COX-2 in the infiltrating monocytes/macrophages at a site of inflammation may regulate resolution of acute inflammation by generating prostaglandins of the J series which would bind to and activate PPAR-γ (296). These findings may have implications for the future treatment of inflammatory conditions. The possibility exists for differential regulation of prostaglandin synthesis at inflammatory sites. A differential regulation of prostaglandin D₂ and E₂ production has been described in murine macrophages (298). Treatment of these cells with TNFα decreased PGD₂ synthesis and increased PGE₂ synthesis (298). Thus selective modulation of the synthesis of pro- and anti-inflammatory prostaglandins may present a new therapeutic option.

Targeting of nuclear receptors may be another means of achieving anti-inflammatory effects without interfering with tissue repair, such as ulcer healing. LT synthesis is initiated at the nuclear envelope (8). This implies that significant levels of LTs are found in the nucleus or deep within the cell. This localization suggests that LTs may have significant autocrine implications such as gene transcription (8). Indeed, an intranuclear receptor for LTB₄, PPARα, has been identified (299). Activation of PPARα results in the induction of genes involved in the oxidation pathways that degrade fatty acids and their derivatives, such as LTB₄ (299). Devchand et al. (299) demonstrated that in vivo, activation of PPARα decreased the duration of an inflammatory response induced by LTB₄ or its precursor arachidonic acid. Preliminary results have demonstrated that the administration of WY14,643, a PPARα activator, in the arachidonic acid-induced murine ear swelling test, is anti-inflammatory, and this is not due to inhibition of 5-LO activity (300). Thus, this may provide an anti-inflammatory option in the future that does not impede ulcer healing.
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APPENDIX

This dissertation is a compilation of the following manuscripts:

