Université de Montréal

Molecular characterization of porcine prostaglandin G/H synthase-2 and its expression in naturally-occurring porcine gastric ulcers

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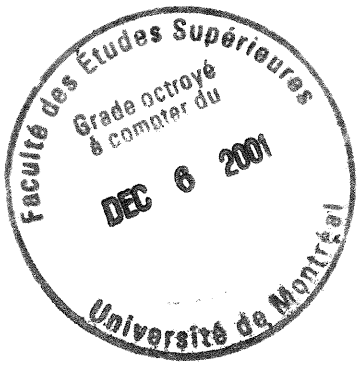

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Ce mémoire intitulé :

Molecular characterization of porcine prostaglandin G/H synthase-2 and its expression in naturally-occurring porcine gastric ulcers

présenté par :

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Résumé

Plusieurs évidences indiquent que l'enzyme prostaglandine G/H synthétase 2 (PGHS-2) participe au processus de réparation des ulcères gastriques. Similairement aux humains, les porcs développent fréquemment des ulcérations gastriques et représentent donc un modèle animal interéssant pour l'étude du processus de réparation des ulcères gastriques. Toutefois, l'expression de la PGHS n'a encore jamais été étudiée chez cette espèce. L'objectif de cette étude était donc de déterminer si les isoformes de la PGHS sont exprimées dans les ulcères gastriques du porc et de caractériser l'ADNc de la PGHS-2 porcine. Des estomacs normaux (n=5) et des ulcères gastriques (n=35) ont été étudiés à l'aide des techniques d'immunohistochimie et de buvardage de type Western en utilisant des anticorps spécifiques contre la PGHS-1 et la PGHS-2. La réaction d'amplification en chaîne par polymérase-transcriptase inverse a été utilisée en vue de caractériser entièrement l'ADNc de la PGHS-2 porcine. Les analyses immunohistochimiques ont révélé que la PGHS-1 était présente dans les estomacs normaux et dans les zones ulcérées. Par contre, alors que la PGHS-2 n'a pas été détectée dans les estomacs normaux, celle-ci était fortement exprimée dans les zones ulcérées de 28 des 35 (80%) ulcères gastriques étudiés ($P < 0.01$). L'analyse par buvardage de type Western a confirmé la présence de la PGHS-2 (apparaissant sous la forme de 2 bandes ayant un poids moléculaire de 72,000/74,000) seulement dans les ulcères gastriques. Les résultats ont aussi démontré que l'ADNc de la PGHS-2

porcine code pour une protéine constituée de 604 acides aminés et présentant une homologie de 89% avec la PGHS-2 humaine. En conclusion, ces résultats révèlent la structure primaire complète de l'enzyme PGHS-2 porcine et démontrent pour la première fois que l'expression de cette enzyme est induite dans les ulcères gastriques du porc.

Mots clés: cyclooxygénase-2, prostaglandine G/H synthétase-2, ulcères gastriques, estomacs, porcs

Summary

Prostaglandin G/H synthase-2 (PGHS-2) is thought to participate in the repair process of gastric ulcerations. Like humans, pigs frequently develop gastric ulcers, and thus represent an attractive animal model to study the repair process of naturally-occurring gastric ulcers. However, the expression of PGHS in the pig stomach has not been reported. The objectives of this study was to determine whether PGHS isoenzymes are expressed in porcine gastric ulcers, and to characterize the porcine PGHS-2 cDNA. Normal stomachs (n=5) and gastric ulcers (n=35) were studied by immunohistochemistry and immunoblot analysis using antibodies selective for PGHS-1 or PGHS-2. Reverse transcription-polymerase chain reaction (RT-PCR) was used to isolate the complete porcine PGHS-2 cDNA. PGHS-1 staining was present in normal stomach and in ulcerated areas. No PGHS-2 was detected in normal stomach, but PGHS-2 was strongly expressed in the ulcerated area in 28 of 35 (80%) gastric ulcers ($P < 0.01$). Immunoblot analysis confirmed the restricted expression of PGHS-2 (doublet with a molecular weight of 72,000/74,000) in ulcerated stomachs. The porcine PGHS-2 cDNA was shown to code for a 604-amino acid protein that is 89% identical to human PGHS-2. These results provide the complete primary structure of porcine PGHS-2, and demonstrate for the first time that the enzyme is induced in naturally-occurring porcine gastric ulcers.

Key words: Cyclooxygenase-2, prostaglandin G/H synthase-2, gastric ulcers, stomach, pigs

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List of abbreviations

COX:	cyclooxygenase
cDNA:	complementary deoxyribonucleic acid
DNA:	deoxyribonucleic acid
EGF:	epidermal growth factor
HGF:	hepatocyte growth factor
kDa:	kilodaltons
IL-1:	interleukin-1
LT:	leukotriene
mRNA	messenger ribonucleic acid
NSAID:	non-steroidal antiinflammatory drug
ORF:	open reading frame
PCR:	polymerase chain reaction
PG:	prostaglandin
PGE_2:	prostaglandin E_2
PGHS:	prostaglandin G/H synthase
PLA_2:	phospholipase A_2
RNA:	ribonucleic acid
RT-PCR:	reverse transcription-polymerase chain reaction
TNF-α:	tumor necrosis factor-α
TxA_2:	thromboxane A_2
3'-UTR:	3'-untranslated region
5'-UTR:	5'-untranslated region

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Introduction

Gastric ulceration is a common condition in pigs that has been associated in part with today's intensive management practices. Porcine gastric ulcers are a cause of substantial economical losses because of sudden deaths or a slowdown in growth rate. Deaths due to gastric ulceration contribute significantly to the total mortality reported in grow-finish pig units. Some of the risk factors involved in porcine gastric ulcers have been identified, but the pathogenesis of this condition is not completely understood. Among the risk factors associated with porcine gastric ulcers, stressful conditions, feed type and size as well as *Helicobacter* spp. infection have been implicated in the etiopathology of this condition. However, little information is currently available on the cellular and molecular mechanisms of gastric ulceration in swine.

Prostaglandins are known to protect the gastric mucosa against damage from injurious agents, and play a role in a multi-level system of protection known as mucosal defense. Prostaglandin G/H synthase (PGHS) is the central enzyme in the formation of prostaglandins. Two isoforms of PGHS exist, and are known as PGHS-1 and PGHS-2. In general, PGHS-1 is constitutively expressed in most tissues, whereas PGHS-2 is usually undetectable in normal tissues, but is induced in many pathologic processes. The role of PGHS isoenzymes in various pathologic conditions, including gastric ulcers, has recently been the subject of several studies in man. Indeed, experimental studies in mice suggest the involvement of PGHS-2 in

the repair process of gastric ulcerations by producing prostaglandins (PG) that are involved in the release of various growth factors implicated in ulcer resolution. The pig represents an interesting animal model for the study of PGHS-2 in gastric ulceration and repair, but no information is currently available on the implication of PGHS-2 in porcine gastric ulcers.

Literature Review

1. Gastric ulcers in pigs

1.1 Economic significance

Gastric ulcers in swine have a worldwide distribution and a high prevalence. In a recent report for the National Pork Producers' Council in the United States, 752 pig stomachs were examined for gastric lesions and severe ulcers were found in 13.7% of them (Morrow et al. 1999). A British study evaluating 1242 porcine stomachs found ulceration in 22.95% (Guise et al. 1997). Furthermore, in a South African survey of more than 4000 pigs, a prevalence of 5.1% of gastric ulcers was reported (Makinde and Gous 1998). Since the 1990's, gastric ulcers have increased in occurrence because of intensive management practices associated with modern swine husbandry (Friendship et al. 2000). Economic losses due to death or growth retardation are easier to determine than the more insidious effects of chronic ulceration (Doster 2000).

1.2 Etiology

Several factors have been implicated in the etiology of gastric ulcers in pigs including diet, race, and stress of transportation (Pfeiffer 1992). Although sex and age do not appear to influence the epidemiology of gastric ulcers, it has been observed that 3-6 month old piglets have the highest rate of

ulceration, and that sows seem particularly at risk around parturition (Friendship 1999). Races bred specifically for rapid growth and lean back fat seem to have higher risk for gastric ulcers, hence suggesting a genetic susceptibility for this condition (Berruecos and Robison 1972). Table I presents a list of potential risk factors associated with ulceration of the *pars esophagea* in pigs.

Table I. Risk factors associated with ulceration of the *pars oesophagea* in pigs.

Nutrition	**Housing/Management**	**Other**
Feed particle size	Confinement rearing	Season
Type of grain	Herd size	Concurrent disease
Milling	Mixing pigs	Parturition
Pelleting	Overcrowding	Heredity
Grinding vs rolling	Holding and transport	Somatotropin
Heat processing	Feeding regimen	Histamine
Lack of fiber		Ascarid infection
Vitamin E/Se deficiency		Helicobacter infection
Rancid fat		
Withdrawal of feed		
Whey		

(source: Friendship, 1999, Gastric ulcers. Diseases of swine: 685-694.)

One of the most important etiological factors seem to be a finely ground diet and pelleting of diets fed to pigs (Hedde et al. 1985; Lawrence et al. 1998). It seems that grow-finish pigs fed cereal grain diet with particle size less than 700 μm have an increased incidence of gastric ulcers (Friendship 1999). Rations containing small feed particles have been suggested to result in increased fluidity and mixing of the stomach contents. This situation could allow gastric irritants like gastric acid and pepsin greater contact with

sensitive oesophageal tissue (Maxwell et al. 1970). Also, increased mixing could cause the gastric pH in the pyloric region to rise, therefore stimulating an increased acid secretion (Friendship 1999). A clear examination of the potential of ulcerogenicity of diet particle size in the absence of other possible risk factors remains to be done.

Irregular feeding patterns due to overcrowding, lack of feeder space, restricted access to water, mistakenly running out of feed, or feeder malfunction have all been linked to gastric ulceration (Friendship 1999; Friendship et al. 2000). Fasting animals for 24 to 72 hours has been shown to dramatically increase the prevalence and severity of ulcers (Friendship 1999). Fasting is speculated to affect gastric pH as fasted pigs treated with omeprazole, a gastric acid pump inhibitor that decreases the amount of acid produced by the stomach, had significantly higher gastric pH. This treatment was effective in preventing some gastric lesions associated with feed withdrawal (Friendship et al. 2000).

The role of *Helicobacter pylori* in the etiology of non-steroidal anti-inflammatory drugs (NSAID)-negative peptic ulcers in humans has been well established (Marshall and Warren 1984). In humans, *H. pylori* infection is present in virtually all patients with duodenal ulcers and in about 70% of patients with gastric ulcers. However, the role of *H. heilmannii*, formerly known as *Gastrospirillum* species (Barbosa et al. 1995; Queiroz et al. 1996; Yeomans and Kolt 1996) and *H. pylori* (Krakowka et al. 1995) as etiological agents of ulceration in swine has not been established. Experimental colonization of pig stomachs with *H. pylori* for as long as 6 months did not

result in gastric ulcers (Eaton et al. 1992; Engstrand et al. 1992). Furthermore, experimental infection of gnotobiotic piglets with a pure culture *of H. heilmannii* also failed to produce gastric ulceration (Krakowka et al. 1998), However, many studies suggest a strong association between the presence of *H. heilmannii* and gastric ulceration in pigs (Barbosa et al. 1995; Queiroz et al. 1996; Roosendaal et al. 2000). For example, it has been reported that approximately 84% to 100% of pigs with gastric ulcers are infected by *H. heilmannii* (Barbosa et al. 1995; Queiroz et al. 1996) in the gastric mucosa. Queiroz et al (1996) noted that *H. heilmannii* type 1 was found in all pigs that had gastric ulcers and in 90% of pigs with preulcerative lesions. In contrast, only 35% of pigs with a normal *pars oesophagea* were *H. heilmannii* type 1 positive. Animals infected with *H. heilmannii* presented different degrees of inflammation in the antral and fundic mucosa.

1.3 Clinical signs

Pigs that die from gastric ulcers are generally in good condition, but show a characteristic pale color. Ulcers can develop rapidly, taking as little as 24 hours to form (Friendship 1999). The main clinical signs are correlated with the extent of blood loss associated with the gastric lesions. Sudden death results from severe blood loss, but paleness, weakness, lethargy, increased respiratory rate and anorexia correlated with anemia can all be manifestations of chronic blood loss. Vomiting can also occur. Rectal temperature of sick animals is generally below normal. Pigs can appear healthy if the ulceration happens without important blood loss.

1.4 Pathogenesis

Gastric ulceration in the pig occurs most commonly in the nonglandular part of the stomach called the *pars oesophagea*, which is similar to the oesophagea in man, without ever affecting the glandular portion of the stomach. The *pars oesophagea* is made up of a stratified squamous epithelium cells and does not secrete cytoprotective mucus (Friendship 1999). Increased hydrochloric acid secretion induced by histamine administration has been noted to be acutely ulcerogenic to the *pars oesophagea* in pigs (Friendship 1999). Compared to other species, the pig has a relatively low gastric motility and the stomach is rarely empty, so mechanical factors could likely be important in the pathogenesis of gastric ulcers (Friendship 1999).

Lesions in the *pars oesophagea* vary in appearance and severity, and usually involve only the mucosa and submucosa. However they may progress to the muscularis externa and, on occasion, to the serosa (Barker et al. 1993). Ulceration ends abruptly at the junction of the glandular portion and the *pars oesophagea*, resulting in a thick area of edematous and inflammatory tissue separating normal and damaged areas (Friendship 1999). In the initial stage of lesion development, the epithelial surface becomes irregular and has a roughened uneven appearance (Doster 2000). Progression of lesion results in erosion followed by ulceration, and hemorrhage is often evident in the submucosa and adjacent mucosa. At this stage, the ulcer either enlarges or heals. In the healing process, fibrous connective tissue proliferates in the *pars eosophagea* (Doster 2000). The

erosive damage to the gastric mucosa can diffuse rapidly, involving the entire pars oesophagea resulting in stenosis of the oesophagus at its entry into the stomach.

2. Mucosal defense of the stomach

Mucosal defense refers to a number of processes that give the gastric mucosa its capacity to resist injury by endogenous secretions, such as acid, pepsin and bile, or by exogenous irritants, like alcohol and NSAIDs. Among these, gastric acid is suggested to be the first line of defense against bacterial colonization of the stomach. Similarly, mucus secreted by the surface epithelium also prevents bacterial adherence and colonization, and is proposed to play a role in protecting the gastric epithelium against acid and pepsin (Figure 1).

The mucus layer is not a static component of mucosal defense, but rather a dynamic element that responds to various agents by increasing in thickness and volume (Bickel and Kauffman 1981; Wallace and Whittle 1986). A study using an *ex vivo* chambered gastric mucosa showed that ethanol treatment resulted in increased mucus production that formed a cap over the gastric mucosa (Wallace and Whittle 1986). Inhibition of epithelial repair was a consequence of physical or chemical removal of this protective mucus cap (Wallace and Whittle 1986).

Bicarbonate is another essential factor in mucosal defense that is directly influenced by increased gastric blood flow in response to mucosal damage (Guttu et al. 1994). Bicarbonate secretion helps create a pH gradient

(Figure 1) that extends from acid in the lumen to near neutrality at the surface of the epithelial cells. Also, other antibacterial substances like antibodies and lactoferrin have gastroprotective roles.

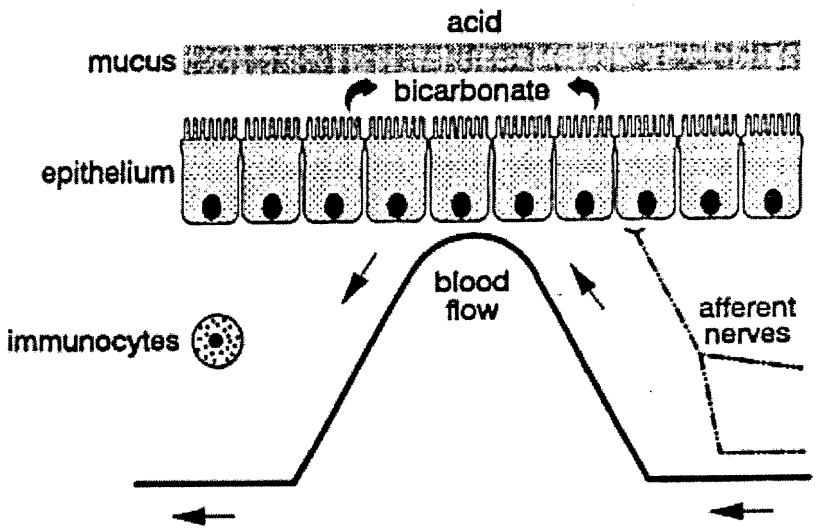

Figure 1. Schematic representation of key components of gastric mucosal protection. The gastric mucosa secretes gastric acid known to play an antibacterial role as well as bicarbonate and a mucus layer. Also, sensory afferent nerves sensitive to acid can initiate a rapid elevation in mucosal blood flow. Immunocytes present in the gastric mucosa can release a variety of vasoactive and chemotactic agents in response to injury that will result in an acute inflammatory reaction. (source: Wallace, *Am J Med* 110: S19-S23., 2001)

Gastric mucosal PGs play a role in mediating the key components underlying mucosal protection. For example, rats that had received A, E and F type PGs orally or subcutaneously had a dose-dependent protection against noxious agents compared to control rats which suffered extensive necrosis to the gastric mucosa (Robert 1979). The ulcerogenic attributes of

NSAIDs are well known, and are thought to be due, in part, to their ability to inhibit cytoprotective PG synthesis, mainly through the inhibition of PGHS-1. Misoprostol, a PGE_1 analog, has been shown to protect and prevent human patients (Graham et al. 1993; Wu et al. 1998) and rats (Bauer et al. 1986) against gastric damage induced by NSAIDs and other injurious agents.

The production of gastroprotective PGs has been attributed to PGHS-1, and these PGs seem to be highly related to the integrity of the gastric mucosa. NS398, a selective PGHS-2 inhibitor, shows little or no inhibition of basal PG synthesis in gastrointestinal tissues (Kargman et al. 1996). However, Langenbach et al. (1995) demonstrated that PGHS-1-knockout mice do not develop spontaneous ulcers, appear to have a normal gastric mucosa, and have a lesser susceptibility to NSAID-induced gastric mucosal damage than the wild-type controls while having approximately 1% of the normal gastric PGE_2 concentrations. Likewise, Morteau et al. (2000) demonstrated that PGHS-1 or –2-knockout mice showed no spontaneous gastrointestinal damage even with nearly undetectable basal intestinal PGE_2 levels in PGHS-1-deficient mice.

Limited studies on the role of PGs in the porcine stomach show that administration of NSAIDs produced damage to the gastric mucosa (Rainsford 1975; 1986). In one of these studies, the authors demonstrated that healthy pigs that received aspirin, which inhibits both PGHS-1 and PGHS-2, and that were exposed to restraint stress developed deep ulcers, whereas only small lesions formed when animals were given aspirin alone. In this study, animals

exposed only to stress displayed no evidence of damage to the gastric mucosa.

3. Arachidonic acid cascade

3.1 Prostanoids

Von Euler first discovered PGs in the 1930s, when it was shown that some vasoactive, lipid-soluble compounds in human seminal fluid and in sheep vesicular gland extracts were able to lower blood pressure in experimental animals (von Euler 1935; 1936; 1983). They were thought to originate from the prostate gland, and were therefore named prostanoids.

Arachidonic acid (AA), a twenty carbon acid, is the major precursor of the eicosanoids which are composed of prostanoids, leukotrienes (LT) and hydroxyeicosatetraenoic (HETE) acid compounds (Figure 2). Within cells, AA is found in an esterified form as a major acyl component of membrane phospholipids (especially phosphatidyl inositol), and its release from phospholipids is thought to be a limiting step in the formation of eicosanoids. The lipid-derived prostanoids consist of PGs and thromboxanes (Tx). These compounds are twenty carbon carboxylic acids, and vary in double bond number placement. PGs contain a cyclopentane ring as well as an α–chain and ω-chain attached to the ring, while Tx contain a cyclohexane ring.

These mediators are not stored by cells, but their synthesis from lipid precursors is triggered by various stimuli. The PGs are a group of hormone-like substances. Like hormones, they play a role in a wide variety of

physiological processes, and act by stimulating target cells into action. However, they differ from hormones in that they largely work locally, in an autocrine or paracrine manner, and are metabolized very rapidly.

Secretory and cytoplasmic phospholipase A_2 ($sPLA_2$ and $cPLA_2$) are activated in response to various physiological and pathological (i.e. inflammation) stimuli, and will cleave the ester bond covalently linking arachidonic acid to the glycerol backbone of the phospholipid precursor. The released arachidonic acid is the main precursor of prostanoid biosynthesis. Because arachidonic acid is the most abundant among precursor fatty acids in most mammals, including humans, the two series of prostanoids are predominantly formed. PGHS, also known as cyclooxygenase (COX), is the central enzyme in the PG biosynthetic pathway (Figure 2). It has two adjacent but distinct active sites (cyclooxygenase and peroxidase). The COX active site first catalyzes the oxygenation of free arachidonic acid to the intermediate, PGG_2, which is then reduced to PGH_2 by the peroxidase activity of the enzyme. PGH_2 is then acted upon by discrete synthases to form the biologically active prostanoids that include PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin), and thromboxane A_2 (TxA_2). PGG_2, PGH_2, PGI_2 and TxA_2 are chemically unstable with short half-lives of 30 seconds to a few minutes (Narumiya et al. 1999), and are degraded into inactive products under normal conditions. PGs are involved in a plethora of physiological processes essential for homeostasis, and also in the pathogenesis of some pathological conditions.

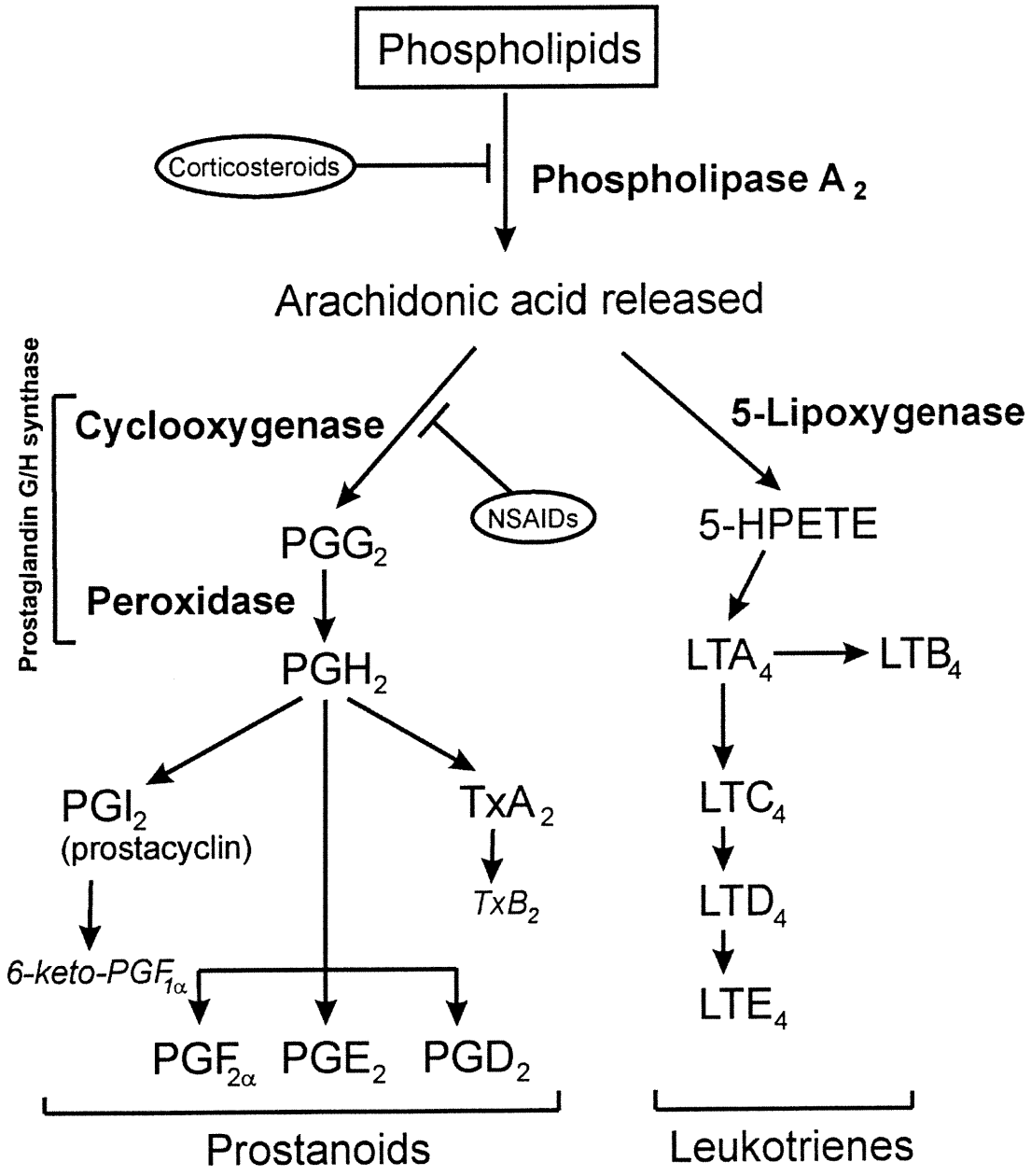

Figure 2. Arachidonic acid cascade with the major eicosanoids. The main enzymes of this biosynthetic pathway are shown in bold. Stable, inactive metabolites of short-lived prostanoids are shown italicized.

The different PGs have been shown to be either vasodilatory or vasoconstrictory, involved in relaxation and contraction of smooth muscles, antisecretory, diarrheogenic, cytoprotective, involved in apoptosis, and oncogenesis, pain and fever, and also recently shown to be implicated in gastric ulcer healing (Mizuno et al. 1997; Shigeta et al. 1998). The major sources and activities of the main prostanoids are summarized in Table II.

3.1.1 Prostanoid receptors

The prostanoid receptors were only recently identified and cloned. They are G protein-coupled receptors that contain seven transmembrane domains. There are eight types and subtypes of prostanoid receptors, which, as a whole, constitute a subfamily in the superfamily of rhodopsin type of receptors. The receptors for PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2 and TxA_2 are named EP (EP_1, EP_2, EP_3, and EP_4), FP, DP, IP and TP, respectively. These receptors exhibit significant cell type-specific expression, and are grouped in categories relevant to their properties, namely relaxant, contractile and inhibitory receptors (Narumiya et al. 1999).

Table II. Summary of the source and physiological responses to the main eicosanoids.

Prostanoid	Primary Source	Physiologic response
TxA_2; TxB_2	Platelets (synthesized by PGHS-1 only)	Vasoconstriction, platelet aggregation
PGI_2	Vascular endothelial cells, macrophages	Vasodilation, inhibits platelet aggregation
PGD_2	Mast cells	Vasodilation, bronchoconstriction
$PGF_{2\alpha}$	Multiple tissues	Vasoconstriction, uterine and bronchial smooth muscle contraction
PGE_2	Leukocytes, other tissue	Vasodilation, inhibits gastric acid secretion, pyrexia, hyperalgesic, inhibits renal tubular reabsorption

In the stomach, EP type receptors seem to be implicated in PGE_2-mediated gastroprotection. Rats administered PGE_2 before being treated with HCl/ethanol to induce gastric lesions were significantly protected against gastric damage, whereas rats administered PGE_2 and EP_1 antagonist were left unprotected to the action of HCl/ethanol treatment. These results suggest a cytoprotective role for PGE_2 via EP_1 (Araki et al. 2000). PGE_2 stimulates bicarbonate secretion via EP_1 in the stomach (Takeuchi et al. 1997), while mucus secretion is mediated by stimulation of the EP_4 receptor in rabbit gastric epithelial cells (Takahashi et al. 1999). PGE_2 plays a role in dilating the arterioles and venules by acting through the EP_2 receptor in rat gastric

mucosal microvasculature, hence improving mucosal blood flow (Ohno et al. 1999).

3.2 Leukotrienes

Eicosanoids also include the LTs which are initially formed by conversion of free AA to 5-HETE by the enzyme 5-lipoxygenase. 5-HETE is further metabolized to LTA_4 then to LTB_4. The latter is known to be a very potent chemoattractant and a promoter of neutrophil adhesion to the endothelium (Asako 1992). LTA_4 can also be metabolized further to LTC_4 and LTD_4 then to LTE_4. LTC_4, LTD_4 and LTE_4 induce strong vasoconstrion and bronchospasms, and increase vascular permeability, leading to augmentation of vascular leakage from venules.

4. PGHS-1 and PGHS-2

4.1 Structure

The mechanism by which aspirin functions was not known until the work of Vane (1971) who proposed that aspirin reduces inflammation primarily by its ability to inhibit prostaglandin production. PGHS was first purified in 1976 (Hemler and Lands 1976) and cloned in 1988 (DeWitt and Smith 1988). In 1991, many groups (Kujubu et al. 1991; O'Banion et al. 1991; Xie et al. 1991) had discovered a second, inducible isoform of the PGHS enzyme, now known as PGHS-2.

PGHS-1 is considered to be the constitutive and PGHS-2 the inducible form of PGHS. The two isoforms have very similar structure and catalytic activity, and both are inhibited by NSAIDs. PGHS-2 originates from an 8.3 kilobase (kb) immediate early gene (Figure 3) found on chromosome 1 (Maier et al. 1990; Herschman 1991), while the PGHS-1 gene, found on chromosome 9, is much larger at 22 kb (Table III). The messenger RNA (mRNA) of PGHS-1 is 2.8 kb in length and PGHS-2 has a 4.0-4.5 kb mRNA (Figure 3). The PGHS-1 open-reading frame (ORF) encodes 602 amino acids, and the PGHS-2 ORF encodes 604 amino acids (Figure 3). The mature, processed PGHS-1 contains 576 amino acids while the mature PGHS-2 includes 587 amino acids.

Both isoforms act as homodimers where each monomer possesses three distinct structural domains: an epidermal growth factor (EGF)-like domain (50 amino acids) at the N-terminus, followed by a membrane binding domain (MBD) (approximately 50 amino acids) (Figure 3), and the much larger C-terminal globular catalytic domain. The MBDs of the PGHS isoforms contain four short amphipathic α helices that extend from the MBD to surround a hydrophobic channel through which fatty acid substrates and NSAIDs are believed to enter the cyclooxygenase active site located in the core of the globular domain (Picot et al. 1994; Kiefer et al. 2000). Protein sequence comparison of the two PGHS isoforms shows approximately 60% identity within the same species (Herschman 1996; Smith and Dewitt 1996). Major amino acid differences between the PGHSs are present in the MBD.

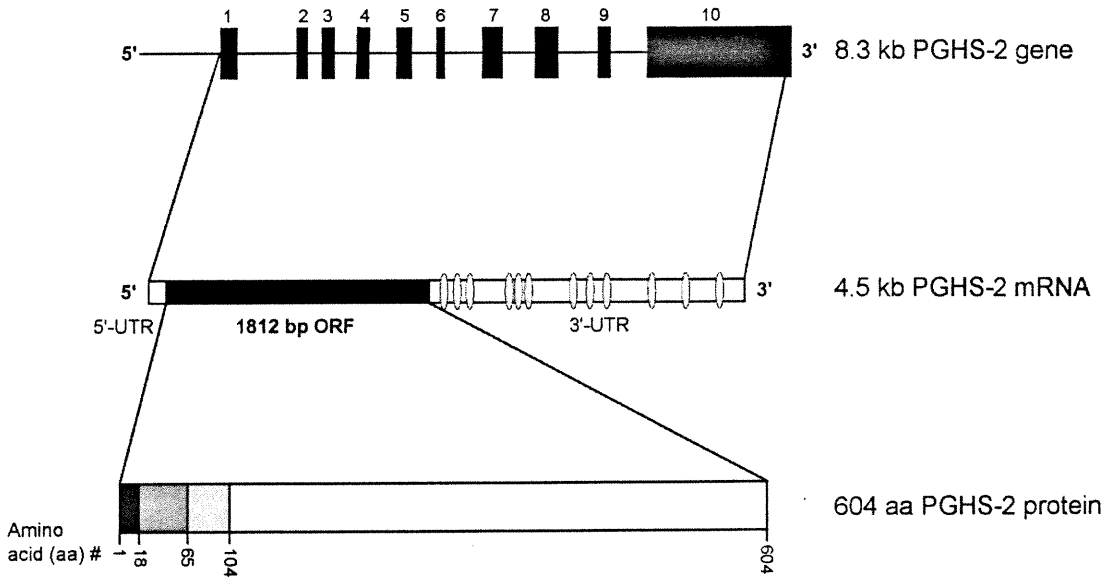

Figure 3. Diagrammatic representation of the 8.3 kb PGHS-2 gene containing 10 exons that encode a 4.5 kb mRNA comprised of a 5'-untranslated region (UTR), a 1812 nucleotide open-reading frame (ORF), followed by a 3'-UTR that harbors several repeats of the Shaw-Kamen motif (AUUUA). The PGHS-2 protein is composed of 604 amino acids. At the N-terminus, the 17 aa signal peptide is followed by an EGF-like domain of more or less 50 aa, then the membrane binding domain which are both of approximately 50 aa. The majority of the protein is composed of the C-terminal globular catalytic domain.

Also, 18 amino acids that are present at the C-terminus of PGHS-2 are absent in PGHS-1. The function of these extra amino acids is not known, as their deletion yields a catalytically active enzyme, and does not affect its subcellular localization (Chan et al, 1995). It is speculated that they might be implicated in the rapid proteolysis of the inducible enzyme (Smith et al. 2000).

Table III. Summary differences between PGHS-1 and PGHS-2

	PGHS-1	PGHS-2
Chromosome	9	1
Gene	22 kb	8.3 kb
messenger RNA	2.8 kb	4.0-4.5 kb
Amino acids	602	604
Protein size following SDS-PAGE	singlet at 69 kDa	doublet at 70-72 kDa
Expression	mostly constitutive	mostly inducible

Interestingly, PGHS-2 is very well conserved among species. All mammals in which PGHS-2 has been cloned and characterized, namely human (Hla and Neilson 1992), cow (Liu et al. 2001), horse (Boerboom and Sirois 1998), sheep (Zhang et al. 1996), mink (Song et al. 1998), mouse (Kujubu et al. 1991), rat (Feng et al. 1993), guinea pig (Bracken et al. 1997), and rabbit (Guan et al. 1997) share more than 80% identity at the nucleic and amino acid levels.

4.2 Intracellular localization and activities

PGHS-1 and PGHS-2 are both integral membrane proteins located in the endoplasmic reticulum and nuclear envelope (Morita et al. 1995). Morita et al. (1995) suggested that PGHS-2 is predominantly associated with the nuclear envelope while PGHS-1 immunoreactivity was found to be equally distributed in the endoplasmic reticulum and the nuclear envelope. They proposed that PGHS-2 metabolites would be favored to the nuclear compartment, which would make them immediately available for the regulation of target genes. A more recent study found that both PGHS-1 and PGHS-2 are present in the same subcellular compartments and at equivalent concentrations (Spencer et al. 1998). Indeed the two isoenzymes were found to be in similar proportions on both the inner and outer membranes of the nuclear envelope as well as on the endoplasmic reticulum in NIH 3T3 cells, human monocytes and human umbilical vascular endothelial cells (HUVECs) (Spencer et al. 1998).

It is currently believed that PGHS-1 and PGHS-2 act independently. As stated above, PGHS-2 could generate prostanoids that play a role in nuclear signaling (Mbalaviele et al. 2000; Harris and Phipps 2001), not by preferential nuclear localization as suggested by Morita et al. (1995), but by differences in expression of the activities of PGHS-1 and PGHS-2 (Spencer et al. 1998). In view of the new findings by Spencer et al. (1998), interactions of PGHS-1 and PGHS-2 with different phospholipases and/or enzyme kinetics are most likely to contribute to the different activities of the two isoenzymes. Two independent pathways using phospholipase A_2 have been proposed, namely

a PGHS-1-dependent transcellular pathway mediated by $sPLA_2$ and a PGHS-2-dependent pathway mediated by intracellular $cPLA_2$ (Reddy and Herschman 1994, 1996). Enzyme kinetic studies of PGHS-1 and PGHS-2 demonstrate that low arachidonic acid concentrations favor PGHS-2-dependent prostaglandin formation whereas high concentrations of exogenous arachidonic acid are associated with a stronger PGHS-1 activity (Swinney et al. 1997; Shitashige et al. 1998). Moreover, PGHS-1 and PGHS-2 seem to utilize different sources of arachidonic acid. *In vitro* models using murine fibroblasts and macrophages have shown that PGHS-2 is necessary for the production of PGs following mitogen or endotoxin stimulation (ligand-induced) while PGHS-1 in these cells cannot utilize the ligand-released arachidonic acid (Reddy and Herschman 1994).

4.3 Expression

The biochemically similar isoenzymes show considerably different patterns of expression. PGHS-1 is shown to be constitutively expressed in nearly all tissues (O'Neill and Ford-Hutchinson 1993; Kargman et al. 1996) where it is thought to synthesize low levels of PGs involved in "housekeeping" functions. In contrast, PGHS-2 mRNA and protein are normally undetectable in most tissues, yet, can be highly induced by numerous agonists, such as proinflammatory cytokines like interleukin (IL)-1α, IL-1β, tumor necrosis factor (TNF)-α (Jones et al. 1993; Ristimaki et al. 1994; Diaz et al. 1998; Longo et al. 1998), lipopolysaccharides (LPS) (Jones et al. 1993; Longo et al.

1998; Barrios-Rodiles et al. 1999), growth factors (Kulkarni et al. 2001), mitogens (Jones et al. 1993; Reddy and Herschman 1994) and intracellular messengers (e.g. cyclic AMP) (Lo et al. 2000). The induced PGHS-2 is responsible for the rise in PGs released locally.

Spencer et al. (1998) have shown that quiescent murine fibroblastic NIH 3T3 cells expressed PGHS-1 and not PGHS-2. However, when these cells were serum-stimulated, PGHS-2 became detectable while PGHS-1 levels stayed the same (Spencer et al. 1998). PGE_2 was the main prostanoid synthesized by serum-starved and serum-stimulated NIH 3T3 cells when supplied with arachidonic acid. PGHS-2 was suggested to be responsible for a 20% increased production of PGE_2 in serum-stimulated NIH 3T3 cells. The authors of this study supported this claim by using specific inhibitors of PGHS-1 and PGHS-2 and a non-selective PGHS inhibitor. Prostanoid synthesis was almost completely blocked in serum-starved 3T3 cells treated with a specific PGHS-1 inhibitor (valerylsalicylate), while the latter caused an 80% inhibition of prostanoid formation in serum-stimulated cells. Use of the specific PGHS-2 inhibitor NS398 resulted in a 15-20% inhibition of prostanoid production in serum-stimulated 3T3 cells, and flurbiprofen which inhibits both PGHS-1 and PGHS-2, completely blocked synthesis in both serum-starved and serum-stimulated 3T3 cells (Spencer et al. 1998).

Comparable results were obtained with murine peritoneal macrophages (PM). Freshly isolated macrophages contained only PGHS-1 and synthesized PGI_2, TxA_2, PGD_2, 12-hydroxyheptadecatrienoic acid (HHT) as well as low amounts of PGE_2, prostanoids that are likely to participate in

"housekeeping" functions. On the other hand, LPS-stimulated PMs expressed PGHS-2 and did not increase expression of all PGHS products, but instead, preferentially metabolized arachidonic acid to PGI_2 and PGE_2 (Brock et al. 1999). This shift in PG production has important repercussions since PGE_2 and PGI_2 have similar effects on cells and tissues.

However, it is now known that this generalization about PGHS-1 and PGHS–2 expression has some exceptions. For example, expression of PGHS-1 could be induced in a phorbol ester-treated THP-1 human monocytic leukemia cell line (Smith et al. 1993), and stem cell factor and dexamethasone induced PGHS-1 in murine bone-marrow-derived mast cells (Samet et al. 1995). Also, PGHS-2 has been shown to be expressed at low levels in many tissues (O'Neill and Ford-Hutchinson 1993; McAdam et al. 1999). Indeed, PGHS-2 is expressed constitutively in tissues of the rat (Yamagata et al. 1993) and piglet brain (Peri et al. 1995), macula densa of rat kidney (Harris et al. 1994), rat testes (McKanna et al. 1998), human tracheal epithelial cells (Walenga et al. 1996), and even in healthy human and rabbit stomach (Zimmermann et al. 1998; Jackson et al. 2000). Iseki also detected weak immunoreactivity for PGHS-2 in surface mucous cells of intact rat stomach (Iseki 1995).

4.4 Regulation of PGHS-2 expression

PGHS-2 can be readily induced in ovarian follicles (Sirois et al. 1992), fibroblasts, monocytes/macrophages and vascular endothelial cells following exposure to LPS, cytokines, growth factors, mitogens, and hormones

(Herschman 1996). These various stimuli are linked to signal transduction pathways that eventually lead to ligand-induced transcriptional activation of the PGHS-2 gene. The promoter regions of the PGHS-2 gene have been cloned in the rat (Sirois and Richards 1993), mouse (Fletcher and Williams 1992), human (Kosaka et al. 1994; Tazawa et al. 1994), and chicken (Xie et al. 1993). The 5' region of the PGHS-2 gene harbors many transcriptional regulatory elements that are apparently used in a cell-specific manner. The characterized PGHS-2 promoters contain the classical TATA box absent from the PGHS-1 promoter (Wang et al. 1993), and experiments using reporter plasmids containing the PGHS-2 5'-region upstream of the transcriptional start site have recognized several specific *cis*-regulatory elements that mediate PGHS-2 gene transcription. Among them, the cyclic AMP response element (CRE) located approximately 60 nucleotides (-60) upstream from the transcriptional start site, the promoter element for the ubiquitous nuclear factor κB (NFκB; -214), and nuclear factor of interleukin-6 (NF-IL6; -124) all have been reported to regulate PGHS-2 gene transcription (Inoue et al. 1994; Kosaka et al. 1994; Inoue et al. 1995).

The CRE is bound and activated by the CCAAT/enhancer binding protein β (C/EBPβ). Xie and Herschman (1995; 1996) found that the CRE in the murine PGHS-2 promoter was essential for the response to the v-*src* oncogene product and serum or platelet-derived growth factor (PDGF).

Using murine mast cells, Reddy et al. (2000) found that the PGHS-2 promoter activity was unaffected when only one of the two NF-IL6 sites was

mutated, but mutation of both NF-IL6 sites led to a substantial loss in promoter activity. Overexpression of the *trans*-acting factor C/EBPβ resulted in increased PGHS-2 promoter activity, while cells cotransfected with dominant negative C/EBPβ constructs had PGHS-2 promoter activity completely blocked, suggesting a critical role for the CRE in PGHS-2 promoter activity (Reddy et al. 2000). Both NF-IL6 and CRE sites are also implicated in the transcriptional activity of the PGHS-2 gene in LPS-and phorbol ester-stimulated endothelium (Inoue et al. 1995). Nakao et al. (2000) have also shown that NFκB is important in IL-1β-induced PGHS-2 gene expression in human gingival fibroblasts.

Although the main level of regulation of the PGHS-2 gene appears to be transcriptional, post-transcriptional regulation of PGHS-2 synthesis also occurs. To demonstrate that PGHS-2 is also regulated at the post-transcriptional level, *in vitro* experiments done in human pulmonary A549 cells (Newton et al. 1998) and IMR-90 cells or synovial fibroblasts (Ristimaki et al. 1996) showed repression of IL-1β-induced PGHS-2 by dexamethasone. Addition of dexamethasone up until 10 h for A549 cells and 16 h for synovial fibroblasts after stimulation with of IL-1β completely inhibited IL1-β–induced PGHS-2 expression. The results of both studies (Ristimaki et al. 1996; Dixon et al. 2000) suggested post-transcriptional mechanisms of dexamethasone-induced mRNA downregulation. This post-transcriptional regulatory system is associated with immediate early genes, and the main mechanism for post-transcriptional control seems to be the targeted and rapid degradation of

mRNA mediated in part by adenylate/uridylate (AU)-rich elements (AREs). The 3'-untranslated region (UTR) of many mRNAs that code for cytokines, proto-oncogenes and nuclear transcription factors are found to be AU-rich, and these mRNAs have short half-lives and are rapidly decayed (Chen and Shyu 1995). Shaw and Kamen (1986) first showed that insertion of an AU-rich portion of the 3'-UTR of the cytokine GM-CSF into the 3'-UTR portion of the β-globin gene could render a stable mRNA transcript highly unstable. They identified a pentanucleotide (AUUUA) present multiple times in the 3'-UTR of GM-CSF as responsible for this instability.

Interestingly, the 3'-UTR of the PGHS-2 mRNA is extremely AU-rich (Dixon et al. 2000), and contains several repeats of the Shaw-Kamen pentanucleotide that appears to mediate its destabilization and short half-life (approximately 1 hour) (Ristimaki et al. 1996; Dixon et al. 2000). However, addition of the PGHS-2 3'-UTR to a heterologous reporter failed to confer dexamethasone sensitivity indicating that this region alone is not enough to give dexamethasone sensitivity (Newton et al. 1998). Constitutively expressed genes like PGHS-1 yield mRNAs that lack these instability elements.

4.5 Role of PGHS-1 and PGHS-2 in inflammation

The use of various selective PGHS-2 inhibitors has contributed to demonstrate the proinflammatory role of this inducible enzyme. Administration of carrageenan to subcutaneous rat air pouch rapidly induces

an inflammatory reaction characterized by fluid exudate and PG synthesis. Masferrer et al. (1994) used this model to show that PGHS-2 mRNA and protein expression in macrophages and fibroblasts paralleled the development of inflammation and PG formation. In this study, indomethacin a nonselective, and NS398, a selective PGHS-2 inhibitor, completely suppressed the formation of proinflammatory PGs in the air pouch (Masferrer et al. 1994). In a carrageenan-induced paw edema model, indomethacin and SC-58125, also a selective PGHS-2 inhibitor, inhibited edema formation and blocked hyperalgesia (Seibert et al. 1994). The induction of locally expressed PGHS-2 mRNA and protein coincided with the production of proinflammatory PGs, edema and hyperalgesia in paw tissue (Seibert et al. 1994). Additionally, using the same model of acute paw inflammation induced by carrageenan injection, edema and hyperalgesia were significantly prevented by pretreatment of the rats with an anti-PGE_2 monoclonal antibody to the same extent as rats receiving indomethacin (Portanova et al. 1996).

The function played by PGHS-1 in inflammation is also being investigated. A study comparing the effects of PGHS-1 and PGHS-2 in carrageenan-induced pleuritis showed that the selective PGHS-2 inhibitors NS398 and nimesulide inhibited inflammation only within the first 2 hours after carrageenan injection and showed no effect by 6 hours (Gilroy et al. 1998). However, treatment with piroxicam and aspirin showed greater inhibition of immunocyte influx, exudate and PGE_2 formation at 2 and 6 hours (Gilroy et al. 1998). Collectively, these results point to a role for PGHS-1 in this model of inflammation.

Also, research by Langenbach and colleagues (1995) has shown that PGHS-1-null mice have a reduced inflammatory response to arachidonic acid, and speculated that a possible reason for this observation could be that arachidonic acid cannot be immediately metabolized to PGH_2 then to PGE_2 by the constitutive PGHS-1. On the other hand, the acute inflammatory response induced by arachidonic acid or tetradecanoyl phorbol acetate (TPA), a potent PGHS-2 inducer, in the ears of PGHS-2-deficient mice is unchanged compared to wild-type animals (Dinchuk et al. 1995; Morham et al. 1995). These studies suggest a possible role for PGs formed by PGHS-1 in the early stage of the inflammatory response. Furthermore, PGHS-2-deficient and wild-type mice had a similar acute inflammatory response to carrageenan-induced paw inflammation, and comparable levels of PGs at the site of inflammation 5 hours after carrageenan injection (Wallace et al. 1998). The study also reports that specific PGHS-2 inhibitors notably inhibited inflammation only when given at doses that caused significant inhibition of PGHS-1.

Some studies propose that PGHS-2 may be critical for a controlled inflammatory response and for its resolution, while PGHS-2 inhibition may lead to an exacerbated inflammatory reaction which could induce greater tissue injury. Mice lacking PGHS-2 showed considerable impairment in mucosal protection to dextran sodium sulfate (DSS)-induced acute colitis compared to normal mice while PGHS-1-deficient mice also demonstrated exacerbation of DSS-induced colonic injury, but to a lesser extent than PGHS-2-null mice. DSS treatment increased intestinal PGE_2 formation in

PGHS-1-deficient and control animals, but not in PGHS-2-null mice (Morteau et al. 2000). Wallace et al. (1998) observed that paw inflammation one week after carrageenan injection had dropped in wild-type mice, whereas paws of PGHS-2-deficient mice were twice the normal size and lymphocyte infiltration persisted. In the inflamed rat colon, PG production has been shown to occur principally through PGHS-2 (Reuter et al. 1996). Indeed, treatment with a highly selective PGHS-2 inhibitor (L-745,337) led to an exacerbation of colitis, and treatment for up to a week resulted in perforation of the colon with 100% mortality in treated animals (Reuter et al. 1996). Another study (Gilroy et al. 1999) reported that rats subjected to carrageenan-induced pleuritis and treated with anti-inflammatory doses of NS398 or indomethacin had a reduced inflammation after 2 hours, but showed a considerably aggravated inflammation after 48 hours. They showed that this exacerbation was associated with reduced PGD_2 and 15-deoxy-Δ^{12-14} PGJ_2 concentrations. The authors proposed that PGHS-2 could be proinflammatory in the early stage of carrageenan-induced pleuritis, but could produce PGs with anti-inflammatory properties at a later phase.

5. Pathophysiology of gastric ulcers

5.1 PGHS and gastric ulcers

Some studies have shown that basal PGHS-1 mRNA and protein are present and not significantly affected in murine models of acetic acid-, acidified ethanol- and ischemia-reperfusion-induced gastric ulceration

(Mizuno et al. 1997; Kishimoto et al. 1998; Shigeta et al. 1998). In contrast, two studies have reported increased PGHS-1 expression in ulcerated human gastric mucosa (Jackson et al. 2000; To et al. 2001). While PGHS-2 expression in the healthy stomach is very low or undetectable (O'Neill and Ford-Hutchinson 1993; Iseki 1995; Mizuno et al. 1997; Sawaoka et al. 1997; Kishimoto et al. 1998), PGHS-2 message and protein are known to be highly induced at sites of gastric tissue damage, with PGHS-2 immunoreactivity usually detected in monocytes/macrophages, fibroblasts and endothelial cells (Schmassmann et al. 1998; Takahashi et al. 1998; Tatsuguchi et al. 2000). Moreover, PGE_2 production is significantly increased in ulcerated areas while absent, or at very low levels, in the surrounding healthy gastric mucosa (Mizuno et al. 1997; Kishimoto et al. 1998; Shigeta et al. 1998; Takahashi et al. 1998). Administration of NS398 has been shown to reduce the increased PGE_2 levels in the ulcerated tissue, but had no effect on basal PGE_2 levels in intact tissues (Shigeta et al. 1998). In the injured mucosa, PGHS-1 also seems implicated in the rise in PGE_2 because indomethacin treatment more potently reduced PGE_2 at the ulcer site than the selective PGHS-2 inhibitor NS398 (Takahashi et al. 1998).

According to a study by Langenbach et al. (1995), PGHS-2 contributes little to PGE_2 production in the stomach of PGHS-1-deficient mice. Likewise, Morteau et al. (2000) showed that colons of PGHS-1-null mice produced no PGE_2 compared with colons of PGHS-2-deficient and wild-type animals. Together, these results suggests against a compensatory pathway, where PGHS-2 could produce compensatory PGs in the absence of PGHS-1.

However, an *ex vivo* study (Kirtikara et al. 1998) reported a compensatory mechanism for PGE_2 production in murine PGHS-1- and PGHS-2-null lung cells. The PGHS-1 deficient cells overexpressed PGHS-2 and $cPLA_2$, while PGHS-2 null cells showed an increased expression of PGHS-1 and $cPLA_2$. The authors of this study suggested that tissue specificity might play a role in the compensatory mechanism they observed, since the studies mentioned above (Langenbach et al. 1995; Morteau et al. 2000) imply against such a mechanism. It is also likely that other cytoprotective mechanisms like nitric oxide synthesis or release of calcitonin gene-related peptide might compensate for the absence of PGs (Tu and Kang 1998; Vane et al. 1998) in PGHS-deficient mice.

PGHS-2 expression has been noted as early as 30 minutes after ethanol ingestion in gastric surface mucous cells (Saika et al. 2000) and 1 hour after aspirin or indomethacin administration in rats (Davies et al. 1997). Others noted the expression of PGHS-2 beginning 6 hours following damage, and reported that it was still elevated after 48 hours, with a peak at 24 hours (Kishimoto et al. 1998). Animal experiments and some clinical studies showed that highly selective PGHS-2 inhibitors do not damage a healthy and intact gastric mucosa (Chan et al. 1995; Schmassmann et al. 1998; Yeomans et al. 1998). Furthermore, a human study comparing the effects of a selective PGHS-2 inhibitor (rofecoxib) versus the unselective NSAID naproxen on gastric mucosal PG production showed that rofecoxib did not have naproxen's ability to reduce gastric PG availability (Wight et al. 2001). However, studies have demonstrated that administration of NSAIDs or

selective PGHS-2 inhibitors affected healing of pre-existing gastric mucosal lesions and greatly delayed their repair. Mizuno et al. (1997) determined that daily administration of NS398 in the early stages of ulceration significantly delayed healing, while NS398 treatment 10 days after the generation of the ulcer failed to influence the course of repair. Schmassmann et al. (Schmassmann et al. 1998) obtained similar results using another selective PGHS-2 antagonist, L-745,337, at doses producing PGHS-2 selective inhibition. Moreover, adding PGE_2 to indomethacin treatment significantly prevented delays in repair seen in indomethacin-only treated rats that were subjected to acetic-acid-induced gastric ulcers (Wang et al. 1989).

In vitro studies have shown that PGs strongly induce the expression of different growth factors, which are associated with wound healing and restitution. Hepatocyte growth factor (HGF) mRNA is synthesized in the stomach and found in cells of the submucosal, muscular or serosal layers of the gastric wall (Kinoshita et al. 1995; Schmassmann et al. 1997). HGF expression is found in human gastric fibroblasts located under the ulcer, and inhibition of HGF considerably affects ulcer healing. PGE_1 and PGE_2 have been shown to strongly induce HGF expression (Bamba et al. 1998; Takahashi et al. 1999). Rising concentrations of HGF in turn trigger PGHS-2 expression by rat gastric epithelial cells, hence elevating PG production locally (Horie-Sakata et al. 1998; Jones et al. 1999). In addition to that, NSAIDs and selective PGHS-2 inhibitors significantly reduced HGF message at the ulcer edge via inhibition of endogenous PG production (Bamba et al.

1998; Horie-Sakata et al. 1998). Artificial wounding of gastric cell monolayers induced PGHS-2 expression in cells located at the edge of the wound, and specific inhibition of PGHS-2 considerably retarded HGF-mediated wound restitution (Horie-Sakata et al. 1998). Epithelial growth factor (EGF), HGF (Tsuji et al. 1995; Schmassmann et al. 1997) and keratinocyte growth factor (KGF) (Kinoshita et al. 1995) receptors are increasingly expressed in undifferentiated epithelial cells at the margin of healing ulcers in the rat mucosa. Consequently, ligand-stimulation of these receptors will lead to proliferation and restitution of the gastric epithelium.

Proinflammatory cytokines also appear to play an important role in the pathogenesis and resolution of gastric ulcers. IL-1β, TNF-α and transforming growth factor (TGF)-β are all expressed in ulcerated gastric tissue (Takahashi et al. 1998). IL-1β and TNF-α have been shown to significantly induce PGHS-2 expression and PGE_2 production in gastric tissues (Bamba et al. 1998; Takahashi et al. 1998). In contrast, PGE_2 seemed to have a negative transcriptional effect on TNF-α, since LPS-stimulated murine macrophages treated with PGE_2 showed an important reduction in TNF-α mRNA expression, but not in IL-α or -β mRNA (Scales et al. 1989; Santucci et al. 1994). A study by Takahashi and colleagues (1998) using a culture of isolated gastric ulcer base reported that PGHS-2 mRNA and PGE_2 production was promoted by inhibiting the action of TGF-β1, and suggested that PGHS-2 mRNA expression could be positively regulated by IL-1β and TNF-α and negatively by TGF-β1. In contrasts, TGF-β has been shown to induce PGHS-

2 mRNA and/or protein and PGE_2 production in many cell types, such as human airway smooth muscle cells (Fong et al. 2000), rat intestinal and lung epithelial cells (Saha et al. 1999) and osteoblastic MC3T3-E1 cells (Pilbeam et al. 1997).

5.2 Relationship between *Helicobacter* spp., PGHS-2 and gastric ulceration

Before 1983, gastric ulcers in humans were attributed to spicy food, acid, stress and lifestyle until Marshall and Warren (1984) discovered spiral shaped microorganisms in the stomach lining of patients with chronic gastritis and peptic ulcers. The Gram-negative, stomach-colonizing spiraled bacterium now known as *Helicobacter pylori* is regarded as the most widespread infection in man. It has a worldwide distribution since 70-90% of the population in developed countries are reported to carry the microorganism (Dunn et al. 1997). The Center for Disease Control stated that approximately two-thirds of the world's population are infected with *H. pylori* and most infected individuals never suffer any symptoms related to the infection (Center for Disease Control). *H. pylori* is now known to be the major etiological agent of gastric and duodenal ulcers in man. *H. pylori* causes more than 90% of duodenal ulcers and more than 80% of gastric ulcers. Besides gastric and duodenal ulcers, *H. pylori* causes chronic active, chronic persistent and atrophic gastritis in adults and children.

Another species of the *Helicobacter* genus, *H. heilmannii*, has been associated with chronic active gastritis and ulcers in humans (Dunn et al. 1997; Dieterich et al. 1998). This microorganism is also found in cats, dogs and pigs (Queiroz et al. 1996; Dunn et al. 1997; Dieterich et al. 1998). In pigs, *H. heilmannii* (Queiroz et al. 1996) and to a minor extent, *H. pylori* (Krakowka et al. 1995), have been suggested to be implicated in the pathogenesis of gastric ulcers. However, *H. heilmannii* colonizes the fundic and pyloric regions of the pig stomach, while the site of ulceration is located in the *pars oesophagea* (Friendship 1999; Doster 2000). Despite this discrepancy, Queiroz and colleagues (1996) showed a strong correlation between the presence of *H. heilmannii* type 1 in the stomach and gastric ulcers of the *pars oesophagea* in pigs.

Interestingly, *H. pylori* infection has been shown to induce PGHS-2 expression and PGE_2 production in the human and gerbil gastric mucosa (Sawaoka et al. 1998; McCarthy et al. 1999; Takahashi et al. 2000; Tatsuguchi et al. 2000) as well as HGF production by human gastric fibroblasts (Takahashi et al. 2000). Indomethacin and NS398 administration did not increase *H. pylori* number, but aggravated hemorrhagic erosion, neutrophil infiltration, lymphoid follicles, and epithelium damage induced by the infection (Takahashi et al. 2000). *H. pylori* infection has also been reported to induce the production of the proinflammatory cytokine TNF-α mRNA in one of three human gastric epithelial cell lines tested (Nakachi et

al. 2000), and IL-1β along with TNF-α protein *in vivo* (Lindholm et al. 1998), both known inducers of PGHS-2 expression.

Objectives

The aim of this project was to determine if PGHS isoenzymes are present in gastric ulcers of pigs, and to characterize the primary structure of the porcine PGHS-2 cDNA.

Methodology

Materials

The citrate phosphate dextrose solution was obtained from Abbott Laboratories (North Chicago, IL). The Vectastain ABC kit was obtained from Vector Laboratories (Burlingame, Ca). Diaminobenzidine tetrahydrochloride was purchased from Sigma-Aldrich (Oakville, On, Canada). Hybond polyvinylidene difluoride membranes (0.45 μm) were obtained from ICN Pharmaceuticals (Montreal, PQ, Canada). The enhanced chemiluminescence (ECL) detection kit was purchased from Amersham Life Sciences (Arlington Heights, IL). Kodak Bio-Max X-ray film was obtained from Eastman Kodak Company (Rochester, NY). TRIzol total RNA isolation reagent, 1 kb DNA ladder and synthetic oligonucleotides were obtained from Gibco BRL (Life Technologies, Gaithersburg, MD). Electrophoretic reagents were obtained from Bio-Rad Laboratories (Richmond, CA). The Prime-a-Gene labeling system, the Access RT-PCR System and pGem-T Easy Vector were purchased from Promega (Madison, WI). The Gene Clean Kit was obtained from Bio 101 (La Jolla, CA). The porcine genomic library was obtained from Clontech (Palo Alto, Ca). [α-^{32}P]dCTP was purchased from NEN Life Science Products (Mississauga, Ontario).

Tissue Samples and Platelet Isolation

Thirty-five cases of gastric ulcers of the pars oesophagea from pigs submitted for a necropsy at the Département de pathologie et microbiologie of the Faculté de médecine vétérinaire (Université de Montréal) were included in the study. All cases were confirmed by examination of hematoxylin-eosin-saffran-stained sections by a veterinary pathologist. Light microscopic examination of Whartin-Starry-stained tissue sections was also done to evaluate the presence of spiral bacteria. Pigs were either brought dead for necropsy or were euthanatized. Five normal pig stomachs (pars oesophagea) used as controls were obtained from a slaughterhouse. All tissues studied were fixed in 10% neutral buffered formalin, whereas fresh samples from normal stomachs and from gastric ulcers were frozen at -70°C for immunoblot analysis.

Porcine platelets were isolated from whole blood collected by venipuncture in anticoagulant (citrate phosphate dextrose solution, Abbott Laboratories). Platelet-rich plasma was isolated by successive centrifugations of the citrated blood for 3 min at decreasing speed (700, 650, and 600*g*), as previously described (Catafalmo and Dodds. 1989). Platelets were recovered from the platelet-rich plasma by centrifugation at 16,000 X *g* for 10 min, and were stored at -70°C. All animal procedures were approved by the institutional animal care and use committee.

Anti-PGHS Antibodies

Two anti-PGHS antibodies (antibodies 8223 and MF243) were used. Affinity-purified polyclonal antibody 8223 was raised in rabbits against ovine PGHS-1, and was shown to be selective for PGHS-1 in various species (Wimsatt et al. 1993; Sirois 1994; Sirois and Dore 1997; Dore et al. 1998; Tremblay et al. 1999). Antibody MF243 was generously provided by Drs. Jilly F. Evans and Stacia Kargman (Merck Frosst Centre for Therapeutic Research, Pointe-Claire-Dorval, Québec). MF243 was raised in rabbits against ovine placental PGHS-2, and its selectivity for PGHS-2 has previously been characterized (Kargman et al. 1995; Tremblay et al. 1999).

Immunohistochemistry

Immunohistochemical staining was performed using the Vectastain ABC kit (Vector Laboratories), as previously described (Dore et al. 1993). Briefly, formalin-fixed tissues were paraffin-embedded, and 3 mm-thick sections were prepared and deparaffined through graded alcohol series. Endogenous peroxidase was quenched by incubating the slides in 0.3% hydrogen peroxide in methanol for 30 min. After rinsing in PBS for 15 min, sections were incubated with diluted normal goat serum for 20 min at room temperature. Primary antibodies diluted in PBS were applied (8223 at 1:100 dilution and MF243 at 1:7,500 dilution), and sections were incubated overnight at 4°C. Control sections were incubated with PBS or with non-immune rabbit serum. After rinsing in PBS for 10 min, a biotinylated goat anti-rabbit antibody (1:222 dilution) was applied, and sections were incubated for 45 min at room temperature. Sections were washed in PBS for 10 min, and

incubated with the avidin DH-biotinylated horseradish peroxidase H reagents for 45 min at room temperature. After PBS wash for 10 min, the reaction was revealed using diaminobenzidine tetrahydrochloride (DAB) as the peroxidase substrate. Sections were counterstained with Gill's hematoxylin stain and mounted. Immunoreactivity was evaluated in a blinded fashion by two independent observers using a grading system where - = no staining, + = 0-10% positive cells, ++ = 11-30% positive cells, and +++ = ≥ 31% positive cells.

Solubilized cell extracts and immunoblot analysis

Solubilized cell extracts were prepared as previously described (Sirois and Dore 1997). Briefly, tissues were homogenized using a Kinematica PT 1200C Polytron Homogenizer (Fisher Scientific, Montréal, PQ, Canada) in ice-cold TED homogenization buffer (50 mM Tris [pH 8.0], 10 mM ethylenediaminetetraacetic acid [EDTA], and 1mM diethyldithiocarbamic acid [DEDTC]) containing 2 mM octyl glucoside, and centrifuged at 30,000 X *g* for 1 h at 4°C. The crude pellets (membranes, nuclei, and mitochondria) were sonicated (8 sec/cycle; 2 cycles) in ice-cold TED sonication buffer (20 mM Tris [pH 8.0], 50 mM EDTA, and 0.1 mM DEDTC) containing 32 mM octyl glucoside. The sonicates were centrifuged at 13,200 X *g* for 20 min at 4°C. The recovered supernatant (solubilized cell extract) was stored at -70°C until electrophoretic analyses were performed. The protein concentration was determined by the method of Bradford (Bio-Rad Protein Assay). Proteins

were resolved by one-dimensional sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE). Hybond polyvinylidene difluoride (PVDF) membranes were briefly immersed in 100% methanol, rinsed in distilled water, then equilibrated in transfer buffer for 10 min before blotting. Proteins were electrophoretically transferred to the membranes. Blocking of membranes was done using 5% nonfat dry milk in 0.1% TTBS (0.1% Tween-20, 10 mM Tris-buffered saline, pH 7.5) for 1h at room temperature, then washed twice for 2 min at room temperature with 0.1% TTBS. After blockage, membranes were incubated with anti-PGHS antibodies diluted in 0.05% TTBS (0.05% Tween-20, 10 mM Tris-buffered saline, pH 7.5) containing 2% nonfat dry milk for 2 h at room temperature. Membranes were incubated with donkey anti-mouse secondary antibody (1:15,000 dilution) for 1h at room temperature. The membranes were washed and the bound secondary antibody was detected using the enhanced chemiluminescence (ECL) detection kit (Amersham Life Sciences). The signal was visualized on Kodak Bio-Max X-ray film.

Cloning and characterization of the porcine PGHS-2 cDNA

The near complete porcine PGHS-2 cDNA was cloned by a combination of three reverse transcription-polymerase chain reactions (RT-PCR) using the Access RT-PCR System (Promega) and following the manufacturer's protocol. Three RT reactions were performed with primers corresponding to the 5'-end, mid-portion and 3'-end of the transcript (antisense primers 2, 4 and 7; Figure 1). RT reactions were performed at

48°C for 45 min on 200 ng of total RNA extracted (TRIzol, Life Technologies) from a pig preovulatory follicle obtained 34 h after intravenous administration of human chorionic gonadotropin (hCG; 1500 IU)(Côté et al. 2001). PCR reactions were performed under the following cycling conditions: 40 cycles of 30 sec denaturation at 94°C, 1 min annealing at 54°C and 2 min elongation at 68°C in an Omnigene TR3 SM5 thermal cycler (Hybaid Limited, Franklin, MA). Porcine PGHS-2 sense and anti-sense primers were designed from exonic sequences of porcine genomic clones or from partial cDNA sequences deposited in GenBank. Following electrophoresis on a 1.0% TAE-agarose gel, the DNA fragments of interest were excised, purified with the Gene Clean Kit (Bio 101) and ligated into the pGEM-T easy vector (Promega) according to the manufacturer's instructions. DNA sequencing was performed on both strands with vector-based primers (T7 and Sp6) using an ABI autosequencer (Applied Biosystems, Foster City, Ca).

Genomic library screening was used to characterize two small regions of porcine PGHS-2 that were not obtained by RT-PCR, namely the first 28 bp of the coding region as well as a 120-bp portion in the 5'-untranslated region (UTR), and a short 38-bp gap present between two PCR fragments (Figure 1). A porcine genomic library (Clontech) was screened following the manufacturer's protocol with a 5' 1.5-kb *Eco*RI fragment of the bovine PGHS-2 cDNA (Liu et al. 2001) that was labeled with [α-^{32}P]dCTP using the Prime-a-Gene labeling system (Promega). Two positive clones (clones 2-1 and 3-1) isolated from an initial screen of 450,000 phage plaques were purified

through secondary and tertiary screening. Sequencing reactions involving clone 2-1 and anti-sense primer 5'-GGA CTT GAG TGG TCC AAA CTG-3', and clone 3-1 and anti-sense primer 5'-CTA TGA CTG CAG CCT TAA ACC-3', were used to characterize the 5'-end of the coding region and the gap region, respectively.

Statistical analysis

The Fisher's exact test was used to compare the frequency of PGHS-2 expression between normal stomachs and gastric ulcers. Statistical analyses were performed using the JMP Software (SAS Institute Inc., Cary, NC).

Article

Lajoie, S., Sirois, J., and Doré, M. Induction of prostaglandin G/H synthase-2 expression in naturally-occurring gastric ulcers in pigs.

Induction of Prostaglandin G/H Synthase-2 Expression in Naturally-Occurring Gastric Ulcers in Pigs

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ABSTRACT

Prostaglandin G/H synthase-2 (PGHS-2) is thought to participate in the repair process of gastric ulcerations. Like humans, pigs frequently develop gastric ulcers, and thus represent an attractive animal model to study the repair process of naturally-occurring gastric ulcers. However, the expression of PGHS in the pig stomach has not been reported. The objective of this study was to determine whether PGHS isoenzymes are expressed in porcine gastric ulcers, and to characterize the porcine PGHS-2 cDNA. Normal stomachs (n=5) and gastric ulcers (n=35) were studied by immunohistochemistry and immunoblot analysis using antibodies selective for PGHS-1 or PGHS-2. Reverse transcription-polymerase chain reaction (RT-PCR) was used to isolate the complete porcine PGHS-2 cDNA. PGHS-1 staining was present in normal stomach and in ulcerated areas. No PGHS-2 was detected in normal stomach, but PGHS-2 was strongly expressed in the ulcerated area in 28 of 35 (80%) gastric ulcers ($P < 0.01$). Immunoblot analysis confirmed the restricted expression of PGHS-2 (doublet with a molecular weight of 72,000/74,000) in ulcerated stomachs. The porcine PGHS-2 cDNA was shown to code for a 604-amino acid protein that is 89% identical to human PGHS-2. These results provide the complete primary structure of porcine PGHS-2, and demonstrate for the first time that the enzyme is induced in naturally-occurring porcine gastric ulcers.

KEYWORDS: Cyclooxygenase-2, prostaglandin G/H synthase-2, gastric ulcers, stomach, pigs.

INTRODUCTION

Gastric ulcer is a prevalent disease, affecting millions of people in the United States each year (11). The pathophysiology of this widespread digestive pathology is complex and still not entirely understood. The spiraled bacteria *Helicobacter pylori* appears to play an important role in a large percentage of cases, while other factors such as the regular use of non-steroidal anti-inflammatory drugs (NSAIDs) and cigarette smoking also contribute to the development of gastric ulcers (31, 44, 45, 47). In pigs, gastric ulcers also represent a widespread problem that annually causes important economical losses (15, 18). The pathogenesis of porcine gastric ulcers is complex, and the major factors contributing to the development of the ulcerative lesions are multiple and diverse (15, 18). Alimentary factors and stressful conditions are among the risk factors that have been associated with gastric ulcers in pigs (15, 18). As for humans, a spiraled bacterium identified in pigs as *Helicobacter heilmanii* has been implicated in the pathogenesis of porcine gastric ulcers (3, 46). Although there are differences between human and porcine gastric ulcers, such as the distinct anatomical localization of the ulcer (38), the pig represents an attractive animal model to study the repair process of naturally-occurring gastric ulcerations.

Prostaglandins play an important role in the mucosal protection of the stomach (1, 71). By acting on mucus and bicarbonate secretion as well as on blood flow, prostaglandins contribute to maintain gastric mucosal integrity.

Prostaglandin G/H synthase (PGHS), also known as cyclooxygenase, is the first rate-limiting enzyme in the biosynthesis of prostaglandins from arachidonic acid (20, 58). PGHS is a homodimer composed of two subunits of about 70,000 daltons and one heme group (36, 68). Two isoforms of prostaglandin G/H synthase have been characterized, namely PGHS-1 and PGHS-2. Both isoforms share similar catalytic activity, as well as important sequence homology at the nucleic acid and amino acid levels (22, 59), but greatly differ in their pattern of expression. PGHS-1 is constitutively expressed in many tissues, and is thought to mediate physiologic responses requiring biosynthesis of prostaglandins. In contrast, PGHS-2 is generally not expressed in most cells, but can be induced in response to inflammatory reactions, growth factors and tumor promoters (22, 70). The molecular characterization of PGHS-2 has been reported in various species, including in humans, rodents, and in various large animal species (4, 5, 17, 21, 23, 35, 40, 60, 73). Although fragments of the porcine PGHS-2 cDNA have been sequenced, the complete characterization of porcine PGHS-2 has not yet been reported.

PGHS-1, which is constitutively expressed in the stomach, is believed to be the isoform mostly responsible for the synthesis of gastric prostaglandins (29). In the normal stomach of humans and animals, most studies found PGHS-2 mRNA and protein to be expressed at low or undetectable levels, and the physiological role of PGHS-2 in normal stomach remains to be defined (27, 29, 48, 61, 74). However, PGHS-2 overexpression has been reported in several different pathological gastric conditions including gastric cancer, gastric infection

with *H. pylori* in humans, experimental ischemia/reperfusion and stress-induced gastric damage in rats, and gastric ulcer healing (19, 27, 33, 42, 43, 48, 50, 53, 61, 64, 65). In the latter, induced PGHS-2 expression was detected at the ulcer margins or in the ulcer bed by immunohistochemistry in gastric ulcers in humans and in experimentally-induced gastric ulcers in rats and mice (27, 43, 53, 64, 65). However, expression of PGHS-1 and PGHS-2 in naturally-occurring gastric ulcerations in animals has never been reported. The objectives of this study were to evaluate the expression of PGHS enzymes in porcine gastric ulcers, to determine their cellular localization, and to characterize the complete primary structure of porcine PGHS-2.

MATERIALS AND METHODS

Materials. The citrate phosphate dextrose solution was obtained from Abbott Laboratories (North Chicago, IL). The Vectastain ABC kit was obtained from Vector Laboratories (Burlingame, Ca). Diaminobenzidine tetrahydrochloride was purchased from Sigma-Aldrich (Oakville, On, Canada). Hybond polyvinylidene difluoride membranes (0.45 μm) were obtained from ICN Pharmaceuticals (Montreal, PQ, Canada). The enhanced chemiluminescence (ECL) detection kit was purchased from Amersham Life Sciences (Arlington Heights, IL). Kodak Bio-Max X-ray film was obtained from Eastman Kodak Company (Rochester, NY). TRIzol total RNA isolation reagent, 1 kb DNA ladder and synthetic oligonucleotides were obtained from Gibco BRL (Life Technologies, Gaithersburg, MD). Electrophoretic reagents were obtained from Bio-Rad Laboratories (Richmond, CA). The Prime-a-Gene labeling system, the Access RT-PCR System and pGem-T Easy Vector were purchased from Promega (Madison, WI). The Gene Clean Kit was obtained from Bio 101 (La Jolla, CA). The porcine genomic library was obtained from Clontech (Palo Alto, Ca). [α-^{32}P]dCTP was purchased from NEN Life Science Products (Mississauga, Ontario).

Tissue Samples and Platelet Isolation. Thirty-five cases of gastric ulcers of the pars oesophagea from pigs submitted for a necropsy at the Département de pathologie et microbiologie of the Faculté de médecine vétérinaire (Université de

Montréal) were included in the study. All cases were confirmed by examination of hematoxylin-eosin-saffran-stained sections by a veterinary pathologist. Light microscopic examination of Whartin-Starry-stained tissue sections was also done to evaluate the presence of spiral bacteria. Pigs were either brought dead for necropsy or were euthanatized. Five normal pig stomachs (pars oesophagea) used as controls were obtained from a slaughterhouse. All tissues studied were fixed in 10% neutral buffered formalin, whereas fresh samples from normal stomachs and from gastric ulcers were frozen at -70°C for immunoblot analysis.

Porcine platelets were isolated from whole blood collected by venipuncture in anticoagulant (citrate phosphate dextrose solution, Abbott Laboratories). Platelet-rich plasma was isolated by successive centrifugations of the citrated blood for 3 min at decreasing speed (700, 650, and 600 *g*), as previously described (9). Platelets were recovered from the platelet-rich plasma by centrifugation at 16,000 X *g* for 10 min, and were stored at -70°C. All animal procedures were approved by the institutional animal care and use committee.

Anti-PGHS Antibodies. Two anti-PGHS antibodies (antibodies 8223 and MF243) were used. Affinity-purified polyclonal antibody 8223 was raised in rabbits against ovine PGHS-1, and was shown to be selective for PGHS-1 in various species (13, 55, 56, 67, 72). Antibody MF243 was generously provided by Drs. Jilly F. Evans and Stacia Kargman (Merck Frosst Centre for Therapeutic Research, Pointe-Claire-Dorval, Québec). MF243 was raised in rabbits against

ovine placental PGHS-2, and its selectivity for PGHS-2 has previously been characterized (30, 67).

Immunohistochemistry. Immunohistochemical staining was performed using the Vectastain ABC kit (Vector Laboratories), as previously described (14). Briefly, formalin-fixed tissues were paraffin-embedded, and 3 µm-thick sections were prepared and deparaffined through graded alcohol series. Endogenous peroxidase was quenched by incubating the slides in 0.3% hydrogen peroxide in methanol for 30 min. After rinsing in PBS for 15 min, sections were incubated with diluted normal goat serum for 20 min at room temperature. Primary antibodies diluted in PBS were applied (8223 at 1:100 dilution and MF243 at 1:7,500 dilution), and sections were incubated overnight at 4°C. Control sections were incubated with PBS or with non-immune rabbit serum. After rinsing in PBS for 10 min, a biotinylated goat anti-rabbit antibody (1:222 dilution) was applied, and sections were incubated for 45 min at room temperature. Sections were washed in PBS for 10 min, and incubated with the avidin DH-biotinylated horseradish peroxidase H reagents for 45 min at room temperature. After PBS wash for 10 min, the reaction was revealed using diaminobenzidine tetrahydrochloride (DAB) as the peroxidase substrate. Sections were counterstained with Gill's hematoxylin stain and mounted. Immunoreactivity was evaluated in a blinded fashion by two independent observers using a grading system where - = no staining, + = 0-10% positive cells, ++ = 11-30% positive cells, and +++ = $\geq$ 31% positive cells.

Solubilized cell extracts and immunoblot analysis. Solubilized cell extracts were prepared as previously described (56). Briefly, tissues were homogenized using a Kinematica PT 1200C Polytron Homogenizer (Fisher Scientific, Montréal, PQ, Canada) in ice-cold TED homogenization buffer (50 mM Tris [pH 8.0], 10 mM ethylenediaminetetraacetic acid [EDTA], and 1mM diethyldithiocarbamic acid [DEDTC]) containing 2 mM octyl glucoside, and centrifuged at 30,000 X g for 1 h at 4°C. The crude pellets (membranes, nuclei, and mitochondria) were sonicated (8 sec/cycle; 2 cycles) in ice-cold TED sonication buffer (20 mM Tris [pH 8.0], 50 mM EDTA, and 0.1 mM DEDTC) containing 32 mM octyl glucoside. The sonicates were centrifuged at 13,200 X g for 20 min at 4°C. The recovered supernatant (solubilized cell extract) was stored at -70°C until electrophoretic analyses were performed. The protein concentration was determined by the method of Bradford (Bio-Rad Protein Assay). Proteins were resolved by one-dimensional sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE). Hybond polyvinylidene difluoride (PVDF) membranes were briefly immersed in 100% methanol, rinsed in distilled water, then equilibrated in transfer buffer for 10 min before blotting. Proteins were electrophoretically transferred to the membranes. Blocking of membranes was done using 5% nonfat dry milk in 0.1% TTBS (0.1% Tween-20, 10 mM Tris-buffered saline, pH 7.5) for 1h at room temperature, then washed twice for 2 min at room temperature with 0.1% TTBS. After blockage, membranes were incubated with anti-PGHS antibodies diluted in 0.05% TTBS (0.05% Tween-20, 10 mM Tris-

buffered saline, pH 7.5) containing 2% nonfat dry milk for 2 h at room temperature. Membranes were incubated with donkey anti-mouse secondary antibody (1:15,000 dilution) for 1h at room temperature. The membranes were washed and the bound secondary antibody was detected using the enhanced chemiluminescence (ECL) detection kit (Amersham Life Sciences). The signal was visualized on Kodak Bio-Max X-ray film.

Cloning and characterization of the porcine PGHS-2 cDNA. The near complete porcine PGHS-2 cDNA was cloned by a combination of three reverse transcription-polymerase chain reactions (RT-PCR) using the Access RT-PCR System (Promega) and following the manufacturer's protocol. Three RT reactions were performed with primers corresponding to the 5'-end, mid-portion and 3'-end of the transcript (antisense primers 2, 4 and 7; Figure 1). RT reactions were performed at 48ºC for 45 min on 200 ng of total RNA extracted (TRIzol, Life Technologies) from a pig preovulatory follicle obtained 34 h after intravenous administration of human chorionic gonadotropin (hCG; 1500 IU) (10). PCR reactions were performed under the following cycling conditions: 40 cycles of 30 sec denaturation at 94°C, 1 min annealing at 54°C and 2 min elongation at 68°C in an Omnigene TR3 SM5 thermal cycler (Hybaid Limited, Franklin, MA). Porcine PGHS-2 sense and anti-sense primers were designed from exonic sequences of porcine genomic clones or from partial cDNA sequences deposited in GenBank. Following electrophoresis on a 1.0% TAE-agarose gel, the DNA fragments of interest were excised, purified with the Gene Clean Kit (Bio 101)

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Statistical analysis. The Fisher's exact test was used to compare the frequency of PGHS-2 expression between normal stomachs and gastric ulcers. Statistical analyses were performed using the JMP Software (SAS Institute Inc., Cary, NC).

RESULTS

Characteristics of pigs with gastric ulcers. The age of pigs with gastric ulcers ranged from one month to four years old, with a mean of 6.9 ± 1.9 months. Of the 35 cases, 23 animals (65.7%) were submitted alive and were euthanatized, 11 pigs (31.4%) were brought dead to the necropsy service, and the information was absent from the necropsy report of one animal. In addition to gastric ulcers, most pigs (n = 30) were suffering from other pathological processes, including respiratory or gastrointestinal infections (n = 28), and polyserositis (n = 5). Examination of Whartin-Starry-stained sections to detect the presence of spiral-shaped bacteria revealed that bacteria morphologically compatible with *Helicobacter* spp. were present in the glandular mucosa adjacent to the ulcer in only one sample. All normal stomachs were negative for spiral-shaped microorganisms.

PGHS expression in normal porcine stomachs. To determine whether PGHS-1 and/or PGHS-2 was expressed under physiological conditions, immunohistochemical staining was performed on normal porcine stomachs (n = 5). Results showed that some PGHS-1 was present in blood vessels (in endothelial cells and in smooth muscles of the media) and in fibroblasts in the submucosa in all normal stomachs (100%; Figure 2*A* and *B*). However, no PGHS-2 expression was detected in normal porcine stomachs (Figure 2*C* and *D*).

PGHS expression in porcine gastric ulcers. PGHS-1 positive cells were found in the ulcer area in 27 out of 35 cases (75%) (Figure 3*E*), while the ulcerated region from 8 cases (25%) contained no PGHS-1-expressing cells. However, in contrast to normal stomachs where no PGHS-2 was detected, 28 of the 35 (80%) gastric ulcers expressed PGHS-2 ($P < 0.01$; Table 1). The extent and intensity of PGHS-2 staining varied between samples (Table I). PGHS-2 immunostaining was predominantly localized in the cytoplasm of fibroblast-like cells that were localized in the granulation tissue proliferating under the ulcerated area (Figure 3, *A-D*). PGHS-2 staining in these cells was often concentrated around the nuclear membrane (Figure 3*B*).

Immunoblotting of PGHS isoforms in porcine gastric tissues. To characterize each PGHS isoform, solubilized cell extracts were prepared from normal stomachs, gastric ulcers and platelets, and proteins analyzed by Western blotting. When a selective anti-PGHS-1 antibody was used, a 69,000 molecular weight (M_r) band was detected in both normal and ulcerated stomachs (Figure 4*A*). A band of identical molecular weight was detected in porcine platelets (Figure 4*A*), and thus corresponded to porcine PGHS-1. When a selective anti-PGHS-2 antibody was used, no signal was detected in normal stomachs, but PGHS immunoreactivity was observed in the two gastric ulcers (Figure 4*B*). Porcine PGHS-2 appeared as a 72,000-74,000 M_r doublet and a small 62,000 M_r band (Figure 4*B*) believed to correspond to a proteolytic fragment, as previously

observed in other species (55, 57). The absence of detectable PGHS-2 in porcine platelets is in keeping with reports in other species (16).

Cloning and characterization of porcine PGHS-2. The near full-length porcine PGHS-2 cDNA sequence was obtained from three RT-PCR reactions designed to generate contiguous DNA fragments (Figure 1). The first fragment (clone 1.3) corresponded to the 5' region of the cDNA and extended from the 29th to the 1334th nucleotide of the coding region. The second fragment (clone 1.0), which overlapped with the first one, contained the remaining portion of the coding region as well as 541 nucleotides of the 3'-untranslated region. Lastly, the third fragment (clone 1.1) corresponded to the 3' region of the cDNA, did not overlap with the second fragment and contained an additional 1.1 kb of 3'-untranslated region. To complete the characterization of the porcine PGHS-2 cDNA, the remaining portion at the 5'-end of the cDNA and the 38-bp gap between clones 1.0 and 1.1 were obtained from genomic clones. Collectively, the porcine PGHS-2 cDNA contains a 5'-untranslated region of 120 bp, an open reading frame of 1815 bp (including the stop codon) and a long 3'-untranslated region of 1686 bp containing multiple repeats of the Shaw-Kamen sequence 5'-ATTTA-3' (n = 12; Figure 5).

The amino acid sequence of pig PGHS-2 was deduced from the coding region of the cDNA, and comparisons were made with the human (23), bovine (40), equine (4), rat (17), mouse (35), rabbit (21), guinea pig (5), mink (60) and ovine (73) homologs (Figure 6). The porcine PGHS-2 coding region encodes a

604-amino acid protein, which is identical in length to all other known mammalian PGHS-2 proteins except for ovine PGHS-2 that has only 603 residues (73). Comparisons between pig PGHS-2 and other mammalian homologs revealed more than 87% identity at the amino acid level, and all important structural and functional domains implicated in PGHS-2 function appear conserved (Figure 6).

DISCUSSION

This study reports for the first time the induced expression of PGHS-2 in naturally-occurring gastric ulcers in pigs. PGHS-2-expressing cells were found in the majority (80%) of ulcerated stomachs, principally in the granulation tissue at the margins and underneath the ulcer. In recent years, several reports on the expression of PGHS-2 in experimentally-induced gastric ulcers in laboratory animals have appeared, but PGHS-2 expression had not yet been documented in naturally-occurring gastric ulcers in animals. In contrast to PGHS-2, PGHS-1 expression was similar in healthy *versus* ulcerated stomachs, in agreement with most previous reports (33, 43, 64).

The implication of PGHS-2 in gastric ulcer healing has emerged in recent years. Mice and rat models of acetic acid- or ethanol-induced gastric ulcers have been used to document the expression of PGHS-2 in ulcerated stomachs (43, 53, 64). In these models, the use of NS-398, a specific PGHS-2 antagonist, inhibited PGE_2 production in the ulcerated tissues, and impaired the healing of ulcers (43, 53, 64). The induced expression of PGHS-2 has also been documented in rat models of ischemia-reperfusion-induced gastric ulcers and in gastric cryoulcers (33, 51). The process of ulcer healing of the gastric mucosa involves the expression of various growth factors, including hepatocyte growth factor (HGF) which appears to be a key player of the repair process in the stomach (32, 62). Interestingly, recent studies have reported a possible association between HGF expression and PGHS-2 expression in gastric

ulcerated tissues, and proposed that the action of HGF is mediated, at least in part, by PGHS-2 (2, 6, 24). Moreover, studies *in vitro* with a rat gastric cell line have shown that HGF can activate the PGHS-2 gene (28). Endogenous interleukin-1 could be a common regulator of these factors as IL-1 has been shown to up-regulate the mRNA expression of various healing-related factors of the gastric mucosa, including HGF and PGHS-2 (2, 63). The present results demonstrating the expression of PGHS-2 in the granulation tissue of the ulcerated area suggest that PGHS-2 expression could also be involved the repair process following gastric ulceration in the porcine species.

In the present study, PGHS-2 expression was not observed in normal porcine stomachs. This finding is in keeping with the current dogma that PGHS-2 is inducible and not constitutively expressed like PGHS-1, and with reports stating that PGHS-2 protein is absent from normal gastric tissues (29, 39, 48). However a controversy exists about the expression of PGHS-2 in normal gastric tissues as some studies have reported that normal gastric mucosa does express constitutive PGHS-2 (26, 27, 74). It was speculated that PGHS-2 might be an important enzyme generating vasodilatory and possibly cytoprotective prostaglandins in the gastric mucosa. The reasons for these conflicting reports about PGHS-2 expression in normal stomach are unknown, but could reflect, in part, differences in the specificity of the antibodies used and/or species-related differences.

The cellular localization of PGHS-2 in ulcerated stomachs was associated with cells that were morphologically compatible with fibroblasts, a finding also in

agreement with previous reports (27, 51, 53). Immunolocalization of PGHS-2 has also been reported in inflammatory cells (macrophages/monocytes and granulocytes) as well as in endothelial cells (27, 51, 53). No PGHS-2 immunoreactivity was found in endothelial cells in this study, but several PGHS-2 positive vascular endothelial cells have been found in the granulation tissue proliferating in bovine gastric ulcerations (M. Doré, J. Sirois, unpublished data), suggesting the presence of potential species-specific differences in the cellular localization of PGHS-2. Studies *in vitro* using human gastric endothelial cells have shown that PGHS-2 expression is induced in gastric endothelial cells during angiogenesis, and that a selective PGHS-2 inhibitor (NS-398) significantly decreased angiogenesis, suggesting that PGHS-2 derived from endothelial cells is involved in gastric ulcer healing (25).

In humans, *H. pylori* has been closely associated with chronic gastritis and peptic ulcer disease (41), and upregulated expression of PGHS-2 has been documented in various *H. pylori*-related pathologies including gastritis, gastric ulcers and premalignant and malignant gastric lesions (19, 27, 42, 50, 61, 65, 66). Although such an association between bacterial infection and gastric ulcers is not as clear in pigs, there is evidence that a spiral bacteria known as *H. heilmanii* could play a role in porcine gastric ulcerations. Pigs with gastric ulcers show a higher incidence of *H. heilmanii* than pigs with healthy stomachs, with 70 to 100% of ulcerated stomachs displaying the bacterium *versus* approximately 30% for normal stomachs (3, 46). The bacterium implicated has been classified as *H. heilmanii* type 1 (7, 49). However, experimental infection of gnotobiotic

pigs with *H. heilmanii* did not induce gastric ulcerations, suggesting that, although the incidence of *H. heilmanii* in ulcerated stomachs is high, its exact pathogenetic role is still unclear (34). The detection of spiraled bacteria in only one case in the present study also questionned the role of bacteria in the pathogenesis of porcine gastric ulcers. However, Quieroz et al.(46) have previously reported that *H. heilmanii* colonizes predominantly the glandular area of the stomach in pigs while ulcerations occur in the nonglandular region. This phenomenon might have contributed to our low number of bacteria-positive samples since the search for spiraled organisms was done on a limited area of the mucosa near the ulcer, and did not include the rest of the gastric mucosa that was not available (as the samples were archival tissue material).

This study is also the first one to document the cloning and characterization of the full-length porcine PGHS-2 cDNA. Comparative analysis showed that the amino acid sequence of porcine PGHS-2 is very similar to that of other mammalian homologs, being 89%, 88%, 87%, 90%, 91%, 91%, 90%, 88%, 87% to human, rat, mouse, horse, cow, ovine, rabbit, guinea pig, and mink PGHS-2, respectively (4, 5, 17, 21, 23, 35, 40, 60, 73). The porcine enzyme includes all known structural and functional domains involved in PGHS-2 activity. These domains include a putative transmembrane region, heme-coordinating histidines 295 and 374, the prostaglandin G/H synthase active-site tyrosine 371, the aspirin acetylation-site serine 516, and four putative *N*-linked glycosylation sites (12, 37, 54, 69). The presence of multiple repeats of the Shaw-Kamen's sequence (5'-ATTTA-3') in the 3'-untranslated region in the porcine PGHS-2

transcript is also a feature reported for PGHS-2 in several other species.[20-28] This motif has previously been shown to be present in several immediate early genes and to confer instability to mRNAs (8, 52).

In summary, our results provide the complete primary structure of porcine PGHS-2, and demonstrate that the enzyme is expressed in the stomach of the majority of pigs with naturally-occurring gastric ulcers, being concentrated in fibroblast-like cells in the granulation tissue proliferating in the ulcerated area. These findings suggest that PGHS-2 is involved in the tissue repair process during gastric ulceration in pigs, and represent the first demonstration of PGHS-2 expression in an animal model of naturally-occurring gastric ulcers.

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Table 1. Characteristics of pigs with gastric ulcers.

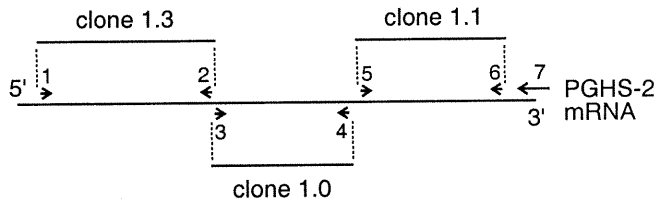
Case	Age (months)	Sex	Death	PGHS-2 Staining*
1	N / D	N / D	N / D	++
2	3	N / D	N	+
3	1.5	N / D	E	-
4	2.4	M	E	+
5	4.8	M	N	+
7	7.2	F	E	+
8	3.6	N / D	N	+
9	3.6	N / D	E	++
10	6	M	N	+
11	48	F	E	+
12	2.4	N / D	N	++
13	2.4	M	N	++
14	2.4	N / D	N	-
15	4.8	F	E	-
16	2	M	N	+++
17	3	F	E	++
18	24	F	N	-
19	2	F	E	-
20	2.4	N / D	E	++
21	48	N / D	E	+++
22	4	N / D	E	+
23	4.8	N / D	E	++
24	4.8	N / D	E	+++
25	1.5	N / D	E	+
26	1	N / D	E	-
27	1.5	N / D	E	+
28	3.6	N / D	E	+++
29	3.6	N / D	N	+
30	3.6	N / D	E	+
31	7.2	N / D	E	-
32	7.2	N / D	E	+
33	6	N / D	E	+
34	3.5	N / D	E	+
35	3.5	N / D	E	+++

p

N / D = not determined, N = natural death, E = euthanasia.

* - = no staining, + = 0-10% positive cells, ++ = 11-30% positive cells, and +++ = ≥31% positive cells.

A



B

1. 5'-CTGCCGTGTCGCTCTGCACTG-3'
2. 5'-TCTCATCTCTCTGCTCTGGTCG-3'
3. 5'-TCGACCAGAGCAGAGAGATGAG-3'
4. 5'-AATAGCAGTCCTGAGCTGAGG-3'
5. 5'-CCCTAACCAAAGGCCTTTAC-3'
6. 5'-TTCCAAATTCCCAGATTCAGGC-3'
7. 5'-GTACCGGATCCTCTAGAGCTCGTCGACCTCGAGGAATTC AAGCTTTTTTTTTTTTTTTTTTT-3'

Figure 1

Figure 1. Cloning strategy for characterizing the porcine PGHS-2 cDNA. (*A*) to obtain a 1.3-kb fragment located at the 5'-end of the cDNA (clone 1.3), RNA was reverse transcribed with primer 2 and the product was subjected to PCR with primers 1 and 2. A 1.0-kb overlapping internal fragment (clone 1.0) was obtained by RT of RNA with primer 4 followed by PCR with primers 3 and 4. A third 1.1-kb fragment corresponding to the 3'-end of the cDNA (clone 1.1) was generated by RT of porcine RNA with primer 6 followed by a first PCR with primers 5 and 7, and a second hemi-nested PCR with primers 5 and 6. (*B*) list of primers used. Specific sense (1, 3, 5) and anti-sense primers (2, 4, 6) were designed from porcine PGHS-2 genomic clones or from partial cDNA sequences deposited in GenBank. Primer 7 was not specific to porcine PGHS-2.

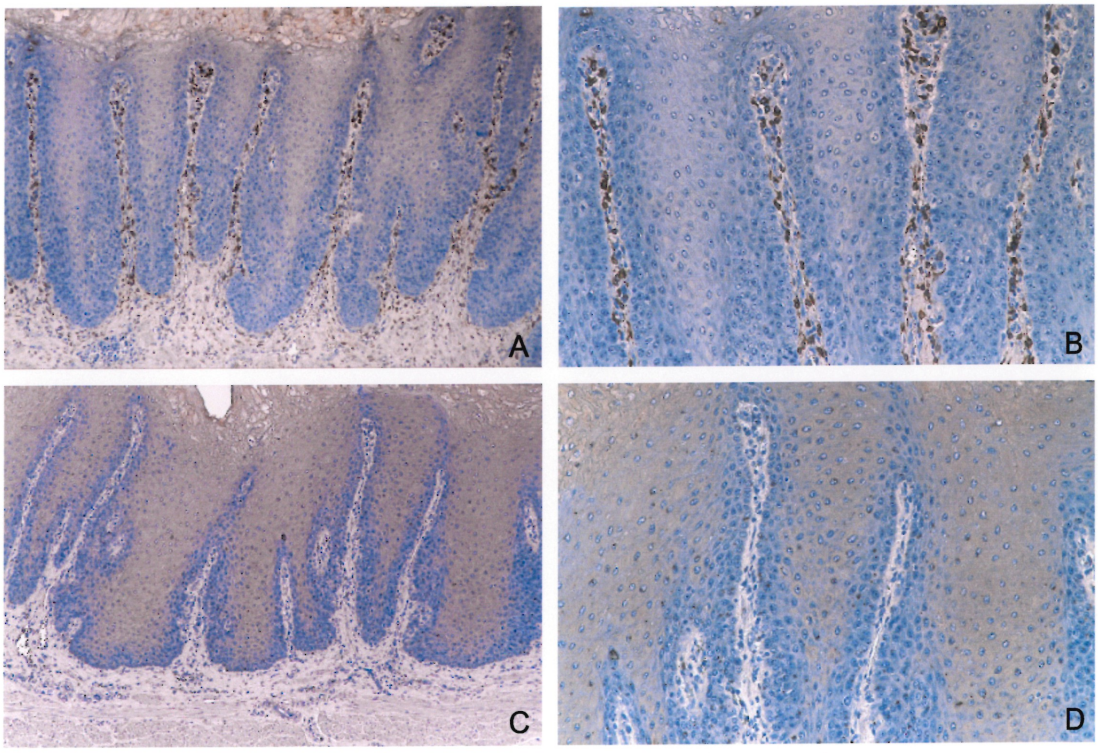


Figure 2

Figure 2. Expression of PGHS-1 and PGHS-2 by normal porcine stomach. Immunohistochemistry was performed on formalin-fixed sections of normal porcine stomachs, as described under *Materials and Methods*. (*A, B*) Immunostaining with antibody 8223 (selective for PGHS-1) demonstrated the presence of numerous PGHS-1-positive cells in the submucosa (*B* is a higher magnification of *A*). (*C, D*) Immunostaining with antibody MF243 (selective for PGHS-2) showed no reactivity in normal stomach (*D* is a higher magnification of *C*). Magnification 200X (*A* and *C*), 400X (*B* and *D*).

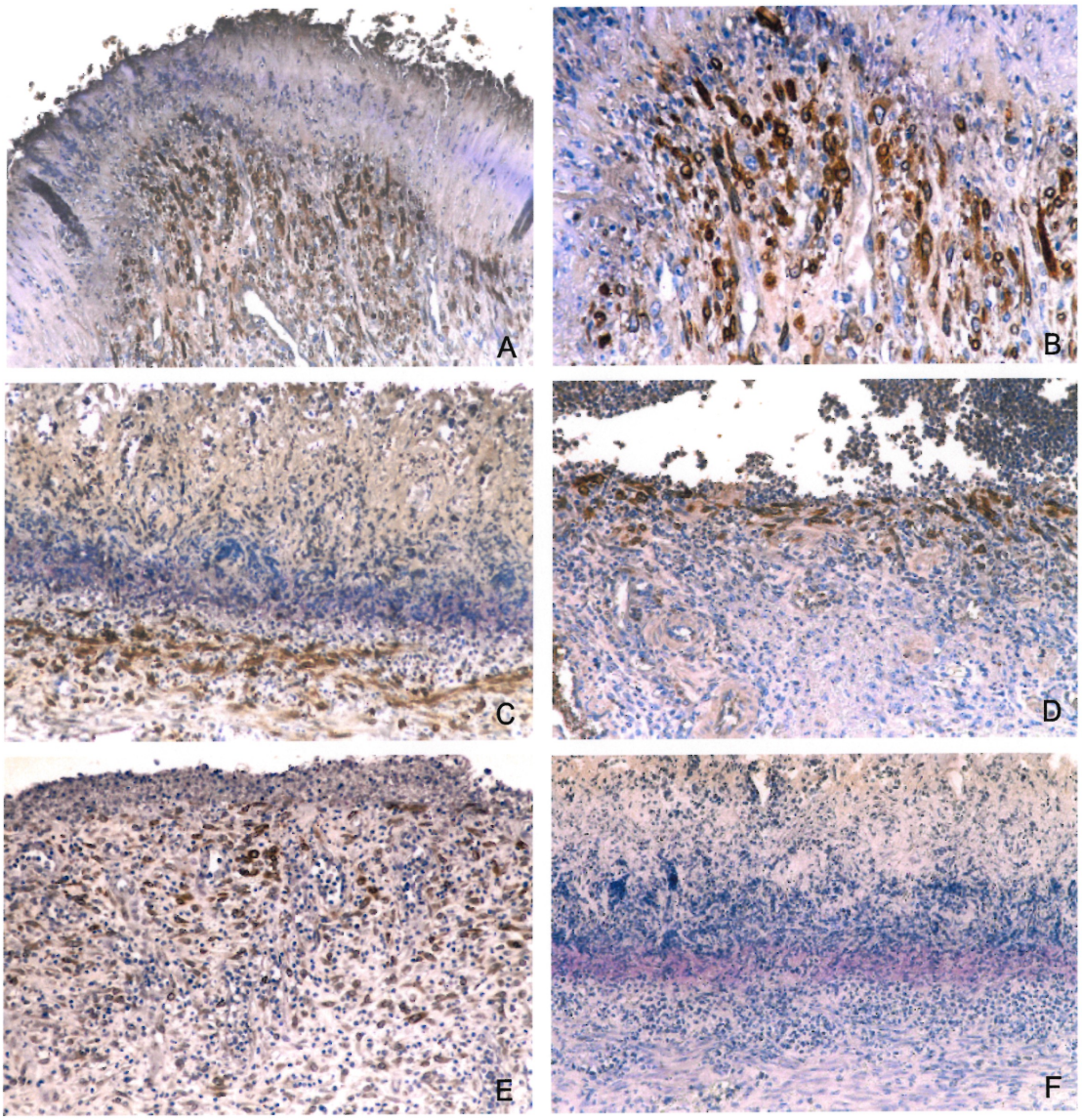


Figure 3

Figure 3. Expression of PGHS-1 and PGHS-2 in porcine gastric ulcers. Immunohistochemistry was performed on formalin-fixed sections of porcine gastric ulcers, as described under *Materials and Methods.* (*A-D*) Immunostaining with antibody MF243 (selective for PGHS-2) revealed the presence of numerous cells staining for PGHS-2 underneath the ulcerated mucosa. Three different gastric ulcers are shown (*A* and *B* are from case 35, *C* is from case 24 and *D* is from case 9, as listed in Table 1). PGHS-2 staining sometimes formed a discrete perinuclear halo (*B*). (*E*) Immunostaining with antibody 8223 (selective for PGHS-1) showed some reactivity in submucosal cells in most gastric ulcers. (*F*) Control staining with normal rabbit serum was negative. Magnification 200X (*A, C-F*), 400X (*B*)

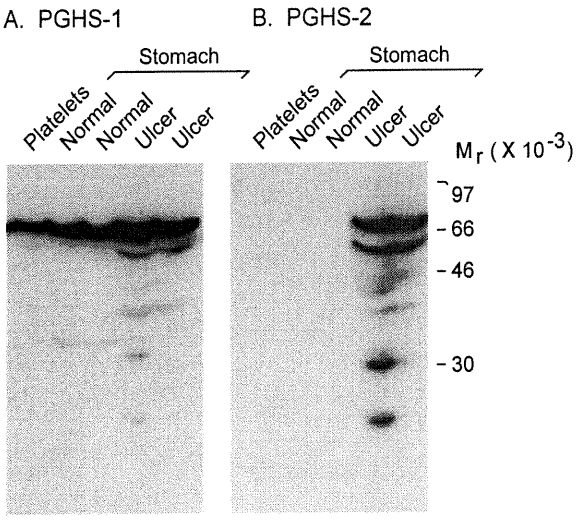

A. PGHS-1
B. PGHS-2
Stomach
Stomach
Platelets
Normal
Normal
Ulcer
Ulcer
Platelets
Normal
Normal
Ulcer
Ulcer
Mr (X 10-3)
97
66
46
30

Figure 4

Figure 4. Immunoblot analysis of PGHS-1 and PGHS-2 isoforms in porcine gastric tissues. Solubilized cell extracts were prepared from normal stomachs, two gastric ulcers (cases 24 and 35; Table 1) and porcine platelets, and were analyzed by one-dimensional SDS-PAGE and immunoblotting, as described under *Materials and Methods*. Duplicate blots were probed with antibody 8223 (selective for PGHS-1, *A*) or antibody MF243 (selective for PGHS-2, *B*). Markers on the right indicate migration of molecular weight standards. Identical amounts of platelets (60 μg), normal stomach (100 μg) and gastric ulcers (100 μg) were loaded in each panel.

```
   1 agcgtcacag gacgcccagg aactcctcac accgcctcct ccagctctac attcagaagc cgactcaccg caacgcctct accagtctgc ctgccagctc
 101 gcgccccgcc cgccgccgag ATGCTCGCCC GCGCCCTGCT GCTCTGCGCT GCCGTGTCGC TCTGCACTGC AGCAAAGCCT TGCTGTTCCA ACCCATGCCA
 201 GAATCGGGGT ATATGTATGA GTGTAGGATT TGACCACTAT AAGTGTGACT GCACCCGAAC AGGATTCTAC GGTGAAAACT GTACTACACC TGAATTTCTG
 301 ACAAGGATAA AATTATTCCT GAAGCCCACT CCAAACACAG TGCACTACAT ACTTACCCAC TTCAAGGGAG TCTGGAACAT TGTCAATAAT ATTCCCTTCC
 401 TGCGGAATGC AATTATGAAA TACGTATTGA TATCGAGATC ACATCTGATT GATAGCCCAC CAACTTACAA TATGCACTAT GGCTATAAAA GCTGGGAAGC
 501 CTTTTCTAAC CTCTCCTATT ATACCAGAGC TCTTCCTCCT GTGCCTGATG ACTGCCCAAC ACCCATGGGT GTGAAAGGGA GGAAAGAGCT TCCCGATTCA
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 901 TACCCGCCTC ACACTCCTGA ACACCTCCGC TTTGCCGTGG GGCATGAGGT CTTTGGTCTG GTGCCTGGTC TGATGATGTA CGCCACAATC TGGCTGCGGG
1001 AACATAATAG AGTGTGCGAT GTGCTTAAAC AGGAGCACCC GGAATGGGAC GATGAACGGC TGTTCCAGAC GAGCAGGCTG ATACTGATAG GAGAAACGAT
1101 TAAGATTGTG ATCGAAGACT ATGTACAACA CCTGAGTGGC TACCACTTCA AACTGAAGTT TGACCCAGAG CTGCTTTTCA ACCAGCAATT CCAATACCAA
1201 AACCGTATTG CTGCTGAGTT TAACACACTC TACCACTGGC ATCCCCTTCT GCCTGACGCC TTCCAGATTG ATGGCCACGA GTACAACTAT CAACAGTTTC
1301 TCTACAATAA CTCTATCTTA CTGGAACATG GCATCACCCA ATTTGTTGAA TCATTTAGCA GGCAAATTGC TGGCAGGGTT GCTGGTGGTA GGAATCTTCC
1401 AGCTGCAGTA CAAAAAGTAT CAAAGGCCTC AATCGACCAG AGCAGAGAGA TGAGATACCA GTCTTTTAAT GAATACCGCA AGCGCTTTCT GCTGAAGCCC
1501 TATCGATCAT TTGAAGAACT TACAGGAGAG AAGGAAATGG CTGCAGAGTT AGAAGCGCTC TATGGTGACA TTGATGCCAT GGAGCTGTAT CCTGCCCTTC
1601 TGGTAGAAAA GCCTCGCCCA GATGCCATCT TTGGGGAGAC CATGGTAGAA GCTGGAGCCC CATTCTCCTT GAAAGGACTT ATGGGTAATC CTATCTGTTC
1701 TCCTGAGTAC TGGAAGCCTA GTACTTTTGG TGGAGAAGTA GGTTTTAAAA TCATCAACAC TGCCTCGATT CAGTCTCTCA TCTGCAATAA TGTGAAAGGC
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2101 ggaaagggga aagcagtttt cattcttttg tacaatccaa tgggaaatga gtgtgacatc tttttacttg aatttcaact tatagtaaga accaaaagct
2201 taagatgttt gaacatttaa atgctgttac aagacggcaa aatgctgcac atttctttct acactgttga tctttccaag gtgtgtgaga aggaacttac
2301 ctactcacca gtacttttag tcgttttgct aagagaaact ctctgctcag tttacttcca tccttttttgt tttcctggtt ttaggatccg agtttgcctt
2401 tctttggact cttgtctctt ttttctacct gaacttttga aagttttcag gaaaacctca gctcaggact gctatttagt tcctcgtaag agggataaag
2501 ggagaaacaa aaaaccctaa ccaaaggcct ttacaaaaag gcatacattc attttgagtg aaagacaaag aactttatcc attgctaatt ttagcaatgt
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3201 ccagaccaca gagaacattg tgtctcatta ggcccggatg gatgttatga gactgacatt ttatacatct tcaaaggacc tgtggatgtt ttgttaattc
3301 attcagccat aatgaattga gaatggagag acattctggg caaagcactg ggttttgata tttttaaatc aagcactgat tacagataac atcagtattt
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Figure 5

Figure 5. Primary structure of the porcine PGHS-2 cDNA. The porcine PGHS-2 cDNA is composed of a 5'-untranslated region of 120 bp (lowercase letters), an open reading frame of 1815 bp (uppercase letters) and a 3'-untranslated region of 1686 bp. The nucleotide sequence was derived from clones isolated by RT-PCR and genomic screening, as described in *Materials and Methods*. The translation initiation (ATG) and stop (TAG) codons are in bold type, Shaw-Kamen motifs (ATTTA) are underlined, and number appearing on the left refer to the first nucleotide on that line. The nucleotide sequence was submitted to GenBank (accession number AY028583).

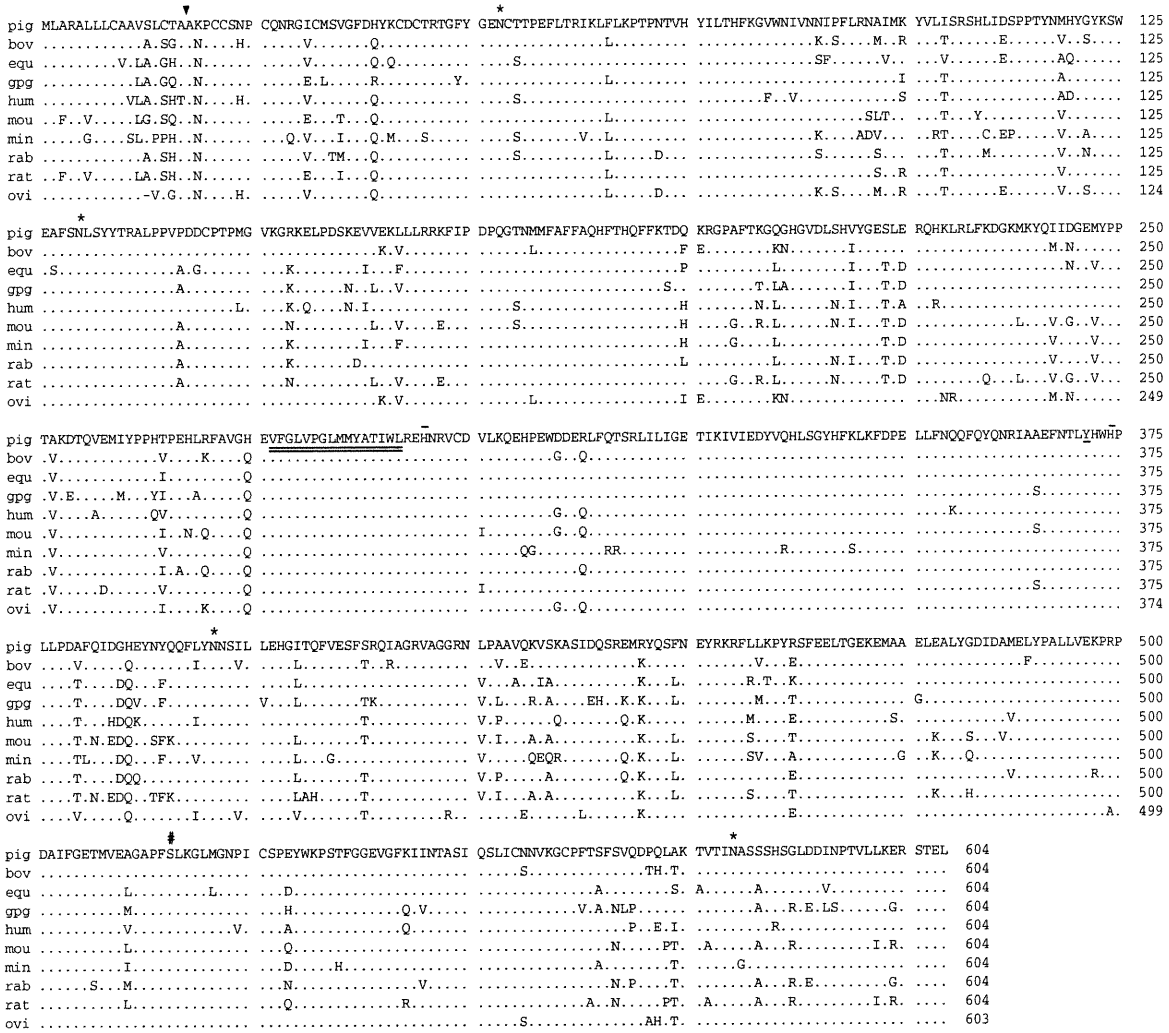

Figure 6

Figure 6. Predicted amino acid sequence of porcine PGHS-2 and comparison with other mammalian homologs. The deduced amino acid sequence of the porcine (por) PGHS-2 is aligned with the human (hum), rat, mouse (mou), bovine (bov), ovine (ovi), equine (equ), rabbit (rab), guinea pig (gpg), and mink (min) homologs. Identical residues are marked with a printed period; the putative signal peptide cleavage site is indicated by an inverse triangle; putative N-glycosylation sites are marked with an asterisk; the putative transmembrane domain is doubled underlined; proximal and distal heme coordination residues are overlined; the tyrosine associated with the prostaglandin G/H synthase active site is underlined and the aspirin-acetylation site is indicated by a number sign. Numbers appearing on the right refer to the last amino acid residue on that line.

Discussion

Previous studies have shown the induction of PGHS-2 in experimentally-induced gastric injury, but PGHS-2 expression had not yet been recorded in naturally-occurring gastric ulcers in animals. Our results show for the first time the induced expression of PGHS-2 in naturally-occurring gastric ulcers in pigs. PGHS-2-expressing cells were found in the majority (80%) of ulcerated stomachs, principally in the granulation tissue at the margins and underneath the ulcer. In contrast to PGHS-2, PGHS-1 expression was similar in healthy and in ulcerated stomachs, in agreement with most previous reports (Mizuno et al. 1997; Kishimoto et al. 1998; Takahashi et al. 1998).

In ulcerated stomachs, PGHS-2 localization was shown in cells that were morphologically compatible with fibroblasts, a finding in agreement with previous reports (Schmassmann et al. 1998; Shigeta et al. 1998; Jackson et al. 2000). Others have reported PGHS-2 immunoreactivity in inflammatory cells (macrophages/monocytes and granulocytes) as well as in endothelial cells (Schmassmann et al. 1998; Shigeta et al. 1998; Jackson et al. 2000). No PGHS-2 immunoreactivity was found in endothelial cells in this study, but several PGHS-2 positive vascular endothelial cells have been found in the granulation tissue proliferating in bovine gastric ulcerations (M. Doré, J. Sirois, unpublished data), suggesting the presence of potential species-specific differences in the cellular localization of PGHS-2. Studies *in vitro*

using human gastric endothelial cells have shown that PGHS-2 expression is induced in gastric endothelial cells during angiogenesis, and that NS398 significantly decreased angiogenesis (Hull et al. 1999), suggesting that PGHS-2 derived from endothelial cells is involved in gastric ulcer healing. In rat models of gastric ulceration, inhibiting selectively PGHS-2 interfered with the process of angiogenesis, and affects ulcer repair (Schmassmann et al. 1998; Shigeta et al. 1998).

Recent evidence supports the implication of PGHS-2 and PGHS-2-formed PGE_2 in gastric ulcer healing. Murine models of cryoulcers and acetic acid- or ethanol-induced gastric ulcers have been used to document the expression of PGHS-2 in ulcerated stomachs (Mizuno et al. 1997; Shigeta et al. 1998; Takahashi et al. 1998). In these models, the use of specific PGHS-2 inhibitors (NS398 and L-745,337) blocked PGE_2 production in the ulcerated tissues, and impaired the healing of ulcers (Mizuno et al. 1997; Schmassmann et al. 1998; Shigeta et al. 1998; Takahashi et al. 1998). The mechanism by which ulcers heal seems to involve the expression of various growth factors in the gastric mucosa, including HGF that appears to have an important role in the repair of gastric lesions (Kinoshita et al. 1995; Takahashi et al. 1996; Schmassmann et al. 1997). Indeed, it was shown that HGF neutralization delayed ulcer healing in rats (Brzozowski et al. 2000). Recent studies have reported a possible interaction between HGF and PGHS-2 expression in gastric ulcerated tissues, and concluded that the action of HGF is mediated, at least in part, by PGHS-2 (Bamba et al. 1998; Horie-Sakata et al. 1998), and that PGHS-2-derived PGs seem responsible for the HGF

upregulation observed (Bamba et al. 1998; Horie-Sakata et al. 1998; Brzozowski et al. 2000). The PGHS-2 gene was shown to be activated by HGF *in vitro* in a rat gastric epithelial cell line (Jones et al. 1999). Endogenous IL-1 could act as a general mediator of these factors as it has been shown to up-regulate the mRNA expression of various healing-related factors of the gastric mucosa, including HGF and PGHS-2 (Bamba et al. 1998; Takahashi et al. 1999). Bamba et al. (Bamba et al. 1998) suggested a IL-1β-PG-HGF pathway in the healing process of gastric ulcers. Moreover, IL-1 (McKean et al. 1995) and HGF (Shen et al. 1997; Tacchini et al. 2000) have been shown to activate NF-κB, and in addition, PGHS-2 was reported to be induced by NF-κB activation in gastric epithelial cells (Kim et al. 2001). Furthemore, prevention of NF-κB activation caused impairment of gastric ulcer healing in rats along with decreased levels of PGHS-2 protein and PGE_2 (Takahashi et al. 2001). The expression of PGHS-2 in the granulation tissue of the ulcerated area shown in the present results suggests that PGHS-2 expression could also be involved the repair process following gastric ulceration in pigs.

In keeping with the current belief that the PGHS-2 enzyme is inducible and not constitutively expressed like PGHS-1, and with studies stating that PGHS-2 protein is absent from normal gastric tissues (Kargman et al. 1996; Ristimaki et al. 1997; Lim et al. 2000), the results of the present study show that PGHS-2 was not present in normal porcine stomachs. However some authors have reported that normal gastric mucosa expresses some

constitutive PGHS-2 (Iseki 1995; Zimmermann et al. 1998; Jackson et al. 2000). It was hypothesized that constitutive PGHS-2 might be important for producing vasodilatory and possibly cytoprotective PGs in the gastric mucosa. The reasons for these contradictory reports about PGHS-2 expression in normal stomach are unknown, but could partly reflect differences in the specificity of the antibodies used and/or species-related differences.

Helicobacter pylori is now known to be the main etiological agent associated with human chronic gastritis and peptic ulcer disease (Marshall and Warren 1984), and PGHS-2 upregulation has been noted in various *H. pylori*-linked pathologies including gastritis, gastric ulcers as well as premalignant and malignant gastric lesions (Sawaoka et al. 1998; Fu 1999; McCarthy et al. 1999; Jackson et al. 2000; Sung et al. 2000; Tatsuguchi et al. 2000; To et al. 2001). Proinflammatory cytokines IL-1β and TNF-α are increased in *H.pylori*-infected subjects (Lindholm et al. 1998), and PGHS-2 is known to be activated by these inflammatory mediators in the stomach (Takahashi et al. 1998). There is some evidence that another species in the *Helicobacter* genus now classified as *H. heilmanii* type 1 (Cantet et al. 1999; Roosendaal et al. 2000) could play a role in porcine gastric ulcerations, although the association between *Helicobacter* infection and gastric ulcers is not clearly defined in pigs. Stomachs of pigs with gastric ulcers show a higher incidence of *H. heilmanii* than pigs with healthy stomachs, with 70 to 100% of ulcerated stomachs positive for this bacterium against approximately 30% for

normal stomachs (Barbosa et al. 1995; Queiroz et al. 1996). Queiroz et al. (Queiroz et al. 1996) concluded to an important relationship between the presence of *H. heilmannii* and porcine gastric ulcers in the *pars oesophagea*. However, despite some evidence for a bacterial origin to porcine gastric ulcers, experimental infection of gnotobiotic pigs with *H. heilmanii* did not induce gastric ulcerations (Krakowka et al. 1998). This finding suggests that, although the occurrence of *H. heilmanii* in ulcerated stomachs is high (Queiroz et al. 1996), its exact role in porcine gastric ulcer pathogenesis is still uncertain. *H. heilmanii* colonizes primarily the glandular area of the stomach in pigs while ulcerations occur in the nonglandular region (Queiroz et al. 1996) Analysis of the gastric ulcers in the present study revealed spiraled bacteria in only one case, argueing against a predominant role for bacteria in the pathogenesis of porcine gastric ulcers. However, the search for spiraled microorganisms was done on a limited area of the mucosa near the ulcer, and did not include the rest of the gastric mucosa that was not available (as the samples were archival tissue material). This might account for our low number of spiraled bacteria-positive samples.

This study is also the first one to document the cloning and molecular characterization of the full-length porcine PGHS-2 cDNA. Comparative analysis showed that the deduced amino acid sequence of the porcine PGHS-2 showed more than 85% identity with that of the human (Hla and Neilson 1992), rat (Feng et al. 1993), mouse (Kujubu et al. 1991), horse (Boerboom and Sirois 1998), cow (Liu et al. 2001), ovine (Zhang et al. 1996), rabbit (Guan et al. 1997), guinea pig (Bracken et al. 1997), and mink (Song et

al. 1998) PGHS-2. The porcine enzyme includes all known structural and functional domains involved in PGHS-2 activity. These domains include a recognized transmembrane region, heme-coordinating histidines 295 and 374, the prostaglandin G/H synthase active-site tyrosine 371, the aspirin acetylation-site serine 516, and four putative *N*-linked glycosylation sites (DeWitt et al. 1990; Shimokawa and Smith 1991; Lecomte et al. 1994; Wennogle et al. 1995). The presence of multiple repeats (n=12) of the Shaw-Kamen's sequence (5'-ATTTA-3') in the 3'-untranslated region in the porcine PGHS-2 transcript is also a feature reported for PGHS-2 in several other species (Kujubu et al. 1991; Hla and Neilson 1992; Feng et al. 1993; Bracken et al. 1997; Guan et al. 1997; Boerboom and Sirois 1998; Song et al. 1998; Liu et al. 2001). This pentanucleotide has previously been shown to be present in several immediate early genes and to confer instability to mRNAs (Caput et al. 1986; Shaw and Kamen 1986) This motif was shown to destabilize the PGHS-2 mRNA and give it its short half-life (Ristimaki et al. 1996; Dixon et al. 2000). The position of several of the Shaw-Kamen sequences varies among species, but interestingly, the first five repeats are consistently found within the first 80 nucleotides downstream of the translation stop codon in all PGHS-2 3'-UTRs (Kujubu et al. 1991; Hla and Neilson 1992; Feng et al. 1993; Bracken et al. 1997; Boerboom and Sirois 1998; Liu et al. 2001). This phenomenon could suggest a relative importance for those five repeats in PGHS-2 mRNA degradation.

In summary, our results provide the complete primary structure of porcine PGHS-2, and demonstrate that the enzyme is expressed in the

stomach of the majority of pigs with naturally-occurring gastric ulcers, being concentrated in fibroblast-like cells in the granulation tissue proliferating in the ulcerated area. These findings suggest that PGHS-2 is involved in the tissue repair process during gastric ulceration in pigs, and represent the first demonstration of PGHS-2 expression in an animal model of naturally-occurring gastric ulcers.

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Conclusion

Porcine gastric ulcers have increased in occurrence because of intensive management practices associated with today's swine husbandry. Although several causal factors for porcine gastric ulcers have been identified, much remains to be elucidated on the pathogenesis and the repair process of this pathology. Gastric ulcers in humans have been shown to be associated with upregulation of PGHS-2, but this had yet to be demonstrated in an animal model of naturally-occurring gastric ulcers.

Under normal conditions, PGHS-1 is expressed at basal levels in gastric tissue, and PGHS-2 is normally undetectable. PGHS-2 is elevated in gastric ulcers, and seems to be implicated in ulcer healing. PGs formed by the induced PGHS-2 contribute to the upregulation of various growth factors involved in wound healing. The induced PGHS-2 in porcine gastric ulcers could play an equal role in ulcer repair as has been found in experimentally-induced gastric lesions. Mounting evidence points to an active involvement of gastric PGs in mucosal protection. Indeed, some preliminary studies have assayed the role of PGs in pig stomachs, and have found NSAIDs to be ulcerogenic (Rainsford 1975; 1986).

Future research could investigate the regulation of PGHS-2 and PG production in cell cultures of porcine gastric fibroblasts through stimulation with various agonists and even using cultures of *H. pylori* or *H. heilmanii*. Study of the porcine PGHS-2 promoter through mutational and transfection

analysis could also yield potentially interesting results. Additionally, the use of specific inhibitors of PGHS-2 in an *in vivo* model of experimentally-induced gastric ulcers in pigs could help to investigate the role of PGHS-2 in this condition. The world of PGHS is still full of discoveries, of which many will be useful in better understanding their role in gastric ulceration and repair.

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