Selective cyclo-oxygenase-2 inhibitors and their influence on the protective effect of a mild irritant in the rat stomach

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1   The effects of the non-selective cyclo-oxygenase (COX) inhibitor indomethacin and the selective COX-2 inhibitors, N-[2-(cyclohexyloxy)-4-nitrophenyl] methanesulphonamide (NS-398), 5-methanesulphonamido-6-(2,4-difluorothio-phenyl)-1-indanone (L-745,337) and 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl) phenyl-2(5H)-furanone (DFU), on the protection induced by the mild irritant 20% ethanol were investigated in the rat stomach.

2   Instillation of 20% ethanol (1 ml, p.o.) effectively protected against gastric mucosal injury induced by subsequent instillation of 70% or 96% ethanol (1 ml, p.o.).

3   Oral administration of indomethacin (1.25–20 mg kg^{-1}) dose-dependently counteracted the protective effect of 20% ethanol (ID_{50}: 3.5 mg kg^{-1}).

4   Likewise, NS-398 (0.1–1 mg kg^{-1}), L-745,337 (0.2–2 mg kg^{-1}) and DFU (0.02–0.2 mg kg^{-1}) inhibited the protective effect of 20% ethanol in a dose-dependent manner with ID_{50} values of 0.3 mg kg^{-1}, 0.4 mg kg^{-1} and 0.06 mg kg^{-1}, respectively.

5   Inhibition of mild irritant-induced protection was also found when NS-398 (1 mg kg^{-1}) was administered s.c. or when 96% ethanol was used to damage the mucosa.

6   Pretreatment with 16,16-dimethyl-prostaglandin (PG)E_2 at 4 ng kg^{-1}, a dose that did not protect against ethanol (70%)-induced mucosal damage when given alone, completely reversed the effect of the selective COX-2 inhibitors on the mild irritant-induced protection.

7   Pretreatment with dexamethasone (3 mg kg^{-1}, 24 and 2 h before instillation of 20% ethanol) did not affect the protective activity of the mild irritant, indicating that enzyme induction is not involved.

8   Indomethacin (20 mg kg^{-1}, p.o.) did not prevent the protection conferred by sodium salicylate (100 mg kg^{-1}), dimercaprol (30 μg kg^{-1}), iodoacetamide (50 mg kg^{-1}) and lithium (20 mg kg^{-1}). Likewise, the protective effect of these agents was not counteracted by NS-398 (1 mg kg^{-1}, p.o.).

9   Whereas indomethacin (20 mg kg^{-1}, p.o.) near-maximally inhibited gastric mucosal formation of PGE_2, 6-keto-$PGF_{1\alpha}$ and thromboxane (TX) B_2 as well as platelet TXB_2 release, the selective COX-2 inhibitors were ineffective.

10   The findings show that selective COX-2 inhibitors, although lacking in ulcerogenic activity, prevent the protection conferred by a mild irritant. Prostaglandis generated by a constitutive COX-2 could thus contribute to physiological functions involved in gastric homeostasis, although at present a non-COX-2-related mechanism underlying the effect of the selective COX-2 inhibitors tested on mild irritant-induced protection cannot be completely excluded.

Keywords: Cyclo-oxygenase-1; cyclo-oxygenase-2; gastroprotection; mild irritant; NS-398; L-745,337; DFU; prostaglandins; thromboxane

Introduction

Prostaglandins are biosynthesized from the precursor fatty acid arachidonic acid. The first step in arachidonic acid metabolism is catalysed by the enzyme cyclo-oxygenase (COX) resulting in bis-oxygenation of arachidonic acid to prostaglandin (PG)G_2 which is further reduced to PGH_2 in a peroxidase reaction by the same protein. Cell-specific isomerization or reduction of PGH_2 by various isomerases or reductases leads to the formation of the different prostaglandins and thromboxane (for review see Smith et al., 1991). Two isoforms of cyclo-oxygenase have been characterized: COX-1 is constitutively expressed in most tissues and has been proposed to catalyse the production of prostaglandins involved in the maintenance of essential physiological functions, such as gastric mucosal integrity, renal function and platelet homeostasis. COX-2 is induced by cytokines, mitogens and endotoxins in inflammatory and other cells and is responsible for the elevated production of prostaglandins that occurs during inflammation and in animal and human tumours (Lee et al., 1992; Xie et al., 1992; Mitchell et al., 1993, 1995; Meade et al., 1993; O'Sullivan et al., 1993; Eberhart et al., 1994; DuBois et al., 1996). Non-steroidal anti-inflammatory drugs block the activity of COX and most drugs currently available for clinical use inhibit both COX-1 and COX-2 with little specificity (Meade et al., 1993; Mitchell et al., 1993). Recently, COX inhibitors with high selectivity for the COX-2 isoform have been developed. In contrast to non-selective COX inhibitors, selective COX-2 inhibitors lack gastric ulcerogenicity (Arai et al., 1993; Masferrer et al., 1994; Chan et al., 1995; Riendeau et al., 1997). This has prompted the hypothesis that only COX-1-derived prostaglandins assist the resistance of the gastric

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mucosa against damage and participate in gastric mucosal defence reactions (Arai *et al.*, 1993; Masferrer *et al.*, 1994; Chan *et al.*, 1995; Vane & Botting, 1995; Riendeau *et al.*, 1997).

Mild irritants such as 20% ethanol protect the gastric mucosa against damage caused by a subsequent exposure to a strong irritant such as concentrated ethanol. This phenomenon has been described as adaptive gastroprotection and has been attributed to stimulation of endogenous prostaglandin formation in the gastric mucosa, since inhibition of prostaglandin biosynthesis by indomethacin abolished the effect (Robert *et al.*, 1983). This study investigates whether the selective COX-2 inhibitors *N*-[2-(cyclohexyloxy)-4-nitro-fenyl]-methanesulphonamide (NS-398) (Arai *et al.*, 1993), 5-methanesulphonamido-6-(2,4-difluorothio-phenyl)-1-indanone (L-745,337) (Chan *et al.*, 1995), 5,5-dimethyl-3-(3-fluorophe-nyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone (DFU) (Riendeau *et al.*, 1997) and dexamethasone mimic the effect of the non-selective COX inhibitor indomethacin. Glucocorticoids have no effect on the activity of COX-1 and COX-2 but prevent the induction of COX-2 by an appropriate stimulus (O'Sullivan *et al.*, 1993). Our findings show that selective COX-2 inhibitors counteract the protective effect of a mild irritant to the same extent as indomethacin, whereas dexamethasone is ineffective. These findings are compatible with the expression of a constitutive COX-2 in the rat gastric mucosa and may indicate that COX-2-derived prostaglandins not only contribute to pathophysiological events but also to essential physiological functions involved in gastric home-ostasis.

This work was presented in part at the 1997 annual meeting of the American Gastroenterological Association in Washington, Washington DC, and published in abstract form (Gretzer *et al.*, 1997).

Methods

Male Wistar rats (weighing 180–220 g) were deprived of food for 24 h with free access to tap water. Rats were kept on a 12-h light-dark cycle and under conditions of controlled temperature ($22\pm1^\circ$C). All experimental protocols were approved by the Animal Care Committee of the Ruhr-University of Bochum.

Protective effect of 20% ethanol

Rats received 1 ml of 20% ethanol by oral intubation followed by oral instillation of 1 ml of 70% or 96% ethanol 30 min later. After a further 5 min, rats were killed by cervical dislocation. The stomach was removed and gross mucosal damage was assessed in a blind manner by calculation of a lesion index by use of a 0–3 scoring system based on the number and severity factor of lesions as described previously (Stroff *et al.*, 1996). The severity factor was defined according to the length of the lesions. Severity factor 0 = no lesions visible; I = lesions <1 mm; II = lesions 2–4 mm; III = lesions >4 mm. The lesion index was calculated as the total number of lesions multiplied by their respective severity factor.

For histological study, a strip of the stomach wall parallel to the limiting ridge was processed by routine methods, stained with H&E and examined under a light microscope. Six rats per group were studied. One full-length portion of tissue across the entire corpus was cut from each stomach. The sections were evaluated in a random, blind fashion by a histologist who was unaware of the experimental protocol.

Two grades of histological injury were assessed: grade 1, superficial damage confined to the surface epithelium; grade 2, deep damage extending beyond the surface epithelium into the region of pits and glands with cells having pyknotic nuclei and hyalinized cytoplasm, loss of normal glandular archi-tecture and cellular dropout. For each stomach strip the length of mucosal areas showing superficial and deep damage was determined and expressed as percentage of the total section length studied.

Effect of indomethacin and selective COX-2 inhibitors on the protection conferred by 20% ethanol

Groups of rats were treated orally with the non-selective COX inhibitor indomethacin (1.25, 5 and 20 mg kg^{-1}) or the selective COX-2 inhibitors NS-398 (0.1, 0.3 and 1 mg kg^{-1}), L-745,337 (0.2, 0.6 and 2 mg kg^{-1}) and DFU (0.02, 0.06 and 0.2 mg kg^{-1}) or dexamethasone (3 mg kg^{-1}). Indomethacin, L-745,337 and dexamethasone were suspended in methylcel-lulose (0.25%). NS-398 was dissolved in absolute ethanol (5 mg ml^{-1}) and was further diluted in saline containing 1% Tween 80. DFU was dissolved in 50 μl dimethylsulphoxide/ Tween 80 (1 : 1 by volume) and further diluted 1 : 20 in saline containing 1% Tween 80. Dilutions of drugs were prepared in the corresponding solvents and administered in a volume of 2.5 ml kg^{-1}. COX-inhibitors were administered for 30 min, dexamethasone 24 and 2 h before instillation of 20% ethanol followed 30 min later by challenge of the mucosa with 70% or 96% ethanol. Controls received the corresponding vehicle. In additional experiments, NS-398 (1 mg kg^{-1}) was administered by s.c. injection 30 min before the instillation of 20% ethanol and subsequent exposure of the mucosa to 70% ethanol. To exclude unspecific damage-modifying effects, all pretreatments used in the study were examined in rats treated with 1 ml of 70% ethanol without pretreatment with 20% ethanol.

Effect of prostaglandin replacement therapy

To investigate whether the effect of selective COX-2 inhibitors on the protection conferred by 20% ethanol is related to inhibition of prostaglandin formation, groups of rats were pretreated with 16,16-dimethyl-PGE$_2$ (4 ng kg^{-1}, dissolved in 70% ethanol (1 mg ml^{-1}) and administered in 0.25% methylcellulose, 2.5 ml kg^{-1}, p.o.) 30 min before administra-tion of NS-398 (1 mg kg^{-1}, p.o.), L-745,337 (2 mg kg^{-1}, p.o.), DFU (0.2 mg kg^{-1}) or indomethacin (20 mg kg^{-1}) followed by oral instillation of 1 ml of 20% ethanol 30 min later. The gastric mucosa was challenged with 1 ml of 70% ethanol 30 min after instillation of the mild irritant. Additional experiments were performed to show that 16,16-dimethyl-PGE$_2$ at a dose of 4 ng kg^{-1} did not protect against gastric mucosal damage induced by a subsequent (60 min) challenge with 1 ml of 70% ethanol.

Effect of inhibition of COX-1 and COX-2 on other types of protection

In additional groups of rats, sodium salicylate (100 mg kg^{-1}), dimercaprol (30 μg kg^{-1}), iodoacetamide (50 mg kg^{-1}) or lithium (20 mg kg^{-1}) were used as protective agents. In these experiments, indomethacin (20 mg kg^{-1}) or NS-398 (1 mg kg^{-1}) were given by gavage 30 min before oral administration of the protective agent followed 30 min later by instillation of 1 ml of 70% ethanol. Mucosal damage was assessed after a further 5 min.

Carrageenin-soaked sponge model of inflammation

Release of PGE_2 into inflammatory exudates was determined to ascertain that with the dose regimens used, dexamethasone prevents induction of COX-2 and NS-398 inhibits COX-2-catalysed prostaglandin formation. Sterile polyester sponges ($1.3 \times 0.7 \times 0.4$ cm), soaked in 2% carrageenin (w/v) in saline, were implanted s.c. to produce an inflammatory response (Higgs *et al.*, 1976). Groups of rats were pretreated orally with dexamethasone (3 mg kg^{-1}, 10 min before) or NS-398 (1 mg kg^{-1}, 30 min before and 4 h after) sponge implantation. Five hours after implantation, the sponges were removed and immersed in 2 ml of phosphate (0.01 M, pH 7.4)-buffered saline containing 5 u ml^{-1} heparin. They were then squeezed, the exudates were immediately centrifuged at $1500 \times g$ at 4°C for 10 min and the supernatants were kept frozen at -80°C until analysis of PGE_2.

Assessment of gastric mucosal formation of PGE_2, 6-keto-$PGF_{1\alpha}$ and TXB_2

Groups of rats were treated orally with 1 ml of 20% ethanol or H_2O. Five, ten or thirty minutes later, the stomach was removed, opened and mucosal fragments from the corpus region were excised, blotted and weighed. To assess effects on gastric mucosal tissue levels of eicosanoids, two 80 mg tissue aliquots were homogenized in 1 ml of ice-cold absolute methanol containing 3 μM indomethacin (to inhibit eicosanoid production during sample handling) using an ultra-turrax T 25 homogenizer (Janke and Kunkel, Staufen, Germany). The methanol was evaporated, the residues were taken up in 1 ml of Tris buffer (50 mM, pH 7.4) and the contents of PGE_2, 6-keto-$PGF_{1\alpha}$ and TXB_2 were determined by radioimmunoassays (RIA). The RIA used are highly specific for the eicosanoid analysed with less than 0.1% crossreaction by other eicosanoids or related compounds. To assess gastric mucosal synthesizing capacity for eicosanoids, two 40 mg tissue aliquots were incubated in oxygenated Tyrode solution at 37°C for 10 min. After the incubation, the medium was removed and analysed for the content of eicosanoids. Additional groups of rats were treated orally with indomethacin (20 mg kg^{-1}), NS-398 (1 mg kg^{-1}), L-745,337 (2 mg kg^{-1}) or DFU (0.2 mg kg^{-1}) 30 min before instillation of 1 ml of 20% ethanol. Controls received the corresponding vehicle. Thirty minutes after instillation of 20% ethanol, the stomach was removed and mucosal fragments were excised, homogenized and extracted or incubated at 37°C as described above.

Assessment of platelet formation of TXB_2

In platelets, TXB_2 is exclusively generated via the COX-1 pathway (Panara *et al.*, 1995). Thus, to assess specifically effects of the compounds studied on the activity of COX-1, release of TXB_2 from platelets during clotting of whole blood was compared in vehicle-treated rats and rats treated with COX inhibitors. Immediately before the stomach was removed, 0.5 ml of blood was collected by cardiac puncture and incubated at 37°C for 60 min. After centrifugation, serum TXB_2 levels were determined by RIA as described in a previous study (Panara *et al.*, 1995).

Assessment of formation of inflammatory PGE_2

The concentration of PGE_2 in the exudates accumulating in the carrageenin-soaked sponges during the 5 h implantation period was determined by RIA.

Statistical analysis

All data are expressed as mean $\pm$ s.e.mean of n values. Comparisons between groups were made by use of Student's t test for unpaired data or the Wilcoxon rank test for non-parametric data. A P value of <0.05 was considered significant.

Drugs

L-745,337 and DFU were kindly supplied by Dr A. W. Ford-Hutchinson (Merck-Frosst Canada, Montreal, Canada). NS-398 was from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). All other chemicals were purchased from Sigma Chemicals Co. (St. Louis, MO, U.S.A.). RIA of PGE_2, 6-keto-$PGF_{1\alpha}$, and TXB_2 were performed by use of anti-eicosanoid-antiplasmen raised in rabbits in our laboratory. ^{3}H-PGE_2 and ^{3}H-6-keto-$PGF_{1\alpha}$ were from New England Nuclear Co. (Dreieich, Germany); [^{3}H]-TXB_2 was from Amersham Buchler (Braunschweig, Germany).

Results

Protection conferred by 20% ethanol and effect of indomethacin and selective COX-2 inhibitors

Oral instillation of 1 ml of 20% ethanol substantially reduced gastric mucosal damage caused by subsequent challenge of the gastric mucosa with 1 ml of 70% ethanol (lesion index 5 ± 1 vs 33 ± 3 in vehicle-treated controls, $P<0.001$, $n=6$ per group). As shown in Figure 1, pretreatment with indomethacin (1.25 – 20 mg kg^{-1}) reversed the protective effect of 20% ethanol in a dose-dependent manner (ID$_{50}$: 3.5 mg kg^{-1}). Likewise, the selective COX-2 inhibitors NS-398 (0.1 – 1 mg kg^{-1}), L-745,337 (0.2 – 2 mg kg^{-1}) and DFU (0.02 – 0.2 mg kg^{-1}) dose-dependently antagonized the protective effect of the mild irritant. ID$_{50}$ values obtained were NS-398 0.3 mg kg^{-1}, L-745,337 0.4 mg kg^{-1} and DFU 0.06 mg kg^{-1}. At the highest doses used, indomethacin, NS-398, L-745,337 and DFU abolished the protective effect of 20% ethanol (lesion index 32 ± 2, 33 ± 3, 27 ± 1 and 33 ± 1, respectively, $P<0.001$ each vs rats treated with 20% ethanol alone, $n=6$ per group). NS-398 (1 mg kg^{-1}) administered by s.c. injection caused identical
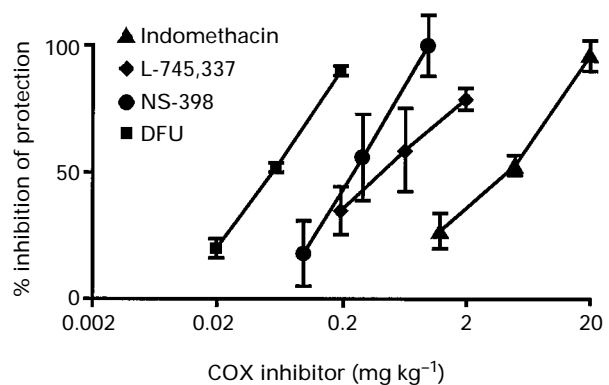

Figure 1 Effect of COX inhibitors on the protection conferred by 20% ethanol. Pretreatment with the non-selective COX inhibitor indomethacin or the COX-2-selective inhibitors NS-398, L-745,337 and DFU inhibited the protective effect of 20% ethanol against mucosal damage caused by 70% ethanol in a dose-dependent manner. Each point represents the mean and vertical lines show s.e.mean of 6 experiments.

inhibition of the mild irritant-induced protection (lesion index 30 ± 2, $n = 6$) thus excluding a nonspecific topical effect. Pretreatment with dexamethasone (3 mg kg^{-1}, 24 and 2 h before administration of the mild irritant) had no effect on the protection conferred by 20% ethanol (lesion index 4 ± 1, $n = 6$) (Figure 2). Dexamethasone given in the absence of 20% ethanol did not protect against mucosal damage caused by 70% ethanol (lesion index 29 ± 5, $n = 6$).

Identical results were obtained when 96% ethanol was used to damage the mucosa. Thus, treatment with 20%, ethanol 30 min before instillation of 96% ethanol reduced the lesion index from 40 ± 1 in controls to 6 ± 1 ($P < 0.001$, $n = 6$ per group). Pretreatment with indomethacin (20 mg kg^{-1}) or NS-398 (1 mg kg^{-1}) substantially inhibited the protective effect of the mild irritant against 96% ethanol (lesion index 33 ± 2% and 34 ± 1%, respectively, $P < 0.001$, $n = 6$ per group). NS-398-sensitive protective effects of 20% ethanol against 96% ethanol were observed as early as 15 min after instillation of the mild irritant (lesion index 8 ± 2 vs 42 ± 1 in H$_2$O-treated control rats, $P < 0.001$, $n = 5$ per group). Pretreatment with dexamethasone (3 mg kg^{-1}) 24 and 2 h before administration of 20% ethanol and followed 30 min later by challenge with 96% ethanol was without effect (lesion index 4 ± 1).

Histological injury

In rats challenged with absolute ethanol, pretreatment with 20% ethanol prevented the development of grade 2 deep histological mucosal injury extending beyond the surface epithelium into the region of pits and glands. Deep histological damage of the mucosa was significantly higher in rats treated with indomethacin (20 mg kg^{-1}) or NS-398 (1 mg kg^{-1}) before 20% ethanol, but not in rats pretreated with dexamethasone (twice 3 mg kg^{-1}) as compared to rats treated with the mild irritant only (Figure 3). The various treatments had no significant effects on the extent of grade 1 superficial damage (data not shown).

Effect of prostaglandin replacement therapy

Oral treatment with 16,16-dimethyl-PGE$_2$ at a dose of 4 ng kg^{-1} did not protect against gastric mucosal damage induced

by instillation of 1 ml of 70% ethanol (lesion index 25 ± 2, $n = 6$). However, 16,16-dimethyl-PGE$_2$ (4 ng kg^{-1}, p.o.) administered 30 min before administration of NS-398 (1 mg kg^{-1}, p.o.), L-745,337 (2 mg kg^{-1}, p.o.) or DFU (0.2 mg kg^{-1}, p.o.) followed by instillation of 20% ethanol and challenge of the gastric mucosa with 70% ethanol, completely reversed the inhibitory effect of the selective COX-2 inhibitors on the mild irritant-induced protection (lesion index 5 ± 1, $n = 4 - 8$, Figure 4). Pretreatment with 16,16-dimethyl-PGE$_2$ in the absence of
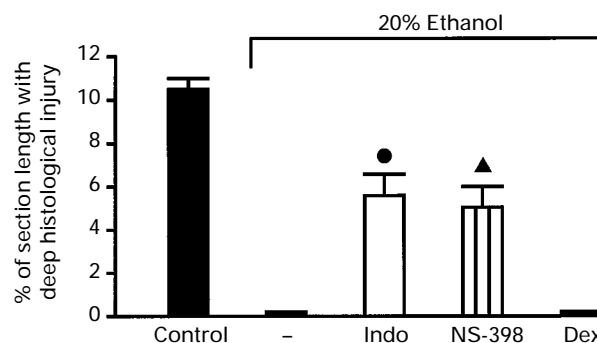
Figure 3 Effects on gastric histology of treatment with indomethacin, NS-398 and dexamethasone in rats exposed to the mild irritant. The length of mucosal areas showed deep mucosal injury was determined and expressed as percentage of the total section length studied per stomach. Rats treated p.o. with indomethacin (Indo, 20 mg kg^{-1}) NS-398 (1 mg kg^{-1}) or dexamethasone (Dex, twice 3 mg kg^{-1}) before oral instillation of 1 ml of 20% ethanol were compared with rats treated with 20% ethanol alone. Mucosal injury was induced by oral instillation of 1 ml of 96% ethanol. Controls were treated with vehicle before instillation of 1 ml of H$_2$O instead of 20% ethanol. All values are expressed as means $\pm$ s.e.mean of 6 experiments in each group. ▲$P < 0.01$; ●$P < 0.002$ vs 20% ethanol alone.

16-DM-PGE2	−	+	−	−	+	−	+	−	+
L-745,337	−	−	−	+	+	−	−	−	−
NS-398	−	−	−	−	−	+	+	−	−
DFU	−	−	−	−	−	−	−	+	+
20% EtOH	−	−	+	+	+	+	+	+	+
70% EtOH	+	+	+	+	+	+	+	+	+

Figure 4 Pretreatment with 16,16-dimethyl-PGE$_2$ (16-DM-PGE$_2$) reversed the effect of NS-398, L-745,337 and DFU on the mild irritant-induced protection. Rats were treated with 16,16-dimethyl-PGE$_2$ (4 ng kg^{-1}, p.o.) 30 min before NS-398 (1 mg kg^{-1}, p.o.), L-745,337 (2 mg kg^{-1}, p.o.) or DFU (0.2 mg kg^{-1}, p.o.) followed by oral instillation of 1 ml of 20% ethanol (ETOH) 30 min later. All rats were challenged with 1 ml of 70% ethanol 30 min after instillation of the mild irritant. Control rats received the corresponding vehicles instead of active drugs or 1 ml of H$_2$O instead of 20% ethanol. Each column represents the mean $\pm$ s.e.mean of $4 - 8$ experiments. *$P < 0.001$ vs rats treated with 70% ethanol in the absence of pretreatment with active agents; ▲$P < 0.001$ vs rats treated with 20% ethanol alone before 70% ethanol; ◆$P < 0.001$ vs rats treated with vehicle before subsequent administration of NS-398, L-745,337 or DFU, 20% ethanol and 70% ethanol.
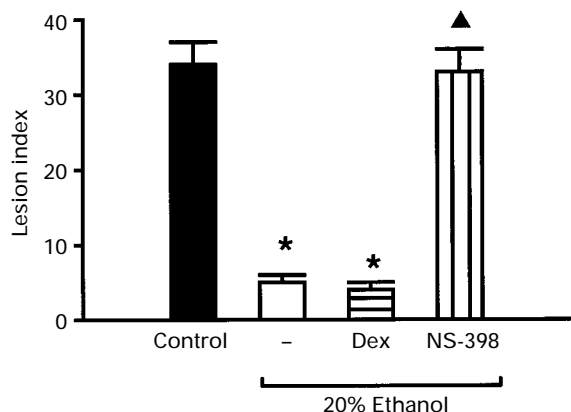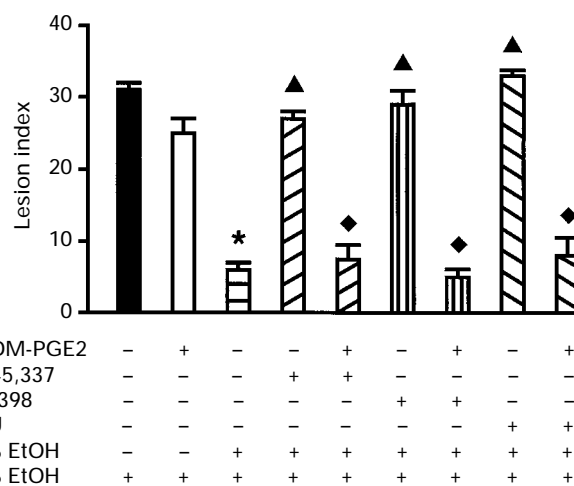
Figure 2 Effect of dexamethasone (Dex) and NS-398 on gastric mucosal protection conferred by 20% ethanol. Rats were pretreated with dexamethasone (3 mg kg^{-1}, p.o., 24 and 2 h) or NS-398 (1 mg kg^{-1}, p.o., 30 min) before oral instillation of 1 ml of 20% ethanol followed by challenge of the gastric mucosa with 1 ml of 70% ethanol 30 min later. Columns represent the mean $\pm$ s.e.mean of $6 - 12$ experiments. *$P < 0.001$ vs controls; ▲$P < 0.001$ vs rats treated with 20% ethanol alone.

selective COX-2 inhibitors did not modify the protective activity of 20% ethanol (lesion index 4 ± 1, $n = 6$). The effect of indomethacin (20 mg kg^{-1}) was not counteracted by 16,16-dimethyl-PGE$_2$ at the dose of 4 ng kg^{-1} (lesion-index 29 ± 4, $n = 6$). Higher doses of the PGE$_2$ analogue could not be tested as they conferred full protection against ethanol-induced mucosal injury when given alone.

Effects of COX inhibitors on the protection conferred by sodium salicylate, dimercaprol, iodoacetamide and lithium

Sodium salicylate (100 mg kg^{-1}), dimercaprol (30 μg kg^{-1}), iodoacetamide (50 mg kg^{-1}), and lithium (20 mg kg^{-1}) conferred near-maximal protection against gastric mucosal damage caused by 70% ethanol. Neither indomethacin (20 mg kg^{-1}) nor NS-398 (1 mg kg^{-1}) attenuated these protective effects. Furthermore, the COX inhibitors did not affect ethanol (70%)-induced gastric mucosal damage in rats treated with vehicle instead of a protective agent (Table 1). Qualitatively identical results were observed with L-745,337 (2 mg kg^{-1}, lesion index 29 ± 4, $n = 4$).

Gastric mucosal and platelet eicosanoids

Gastric prostaglandins and thromboxane: mucosal tissue levels Thirty minutes after oral instillation of 1 ml of 20% ethanol, gastric mucosal levels of PGE$_2$ (119 ± 22 pg mg^{-1}), 6-keto-PGF$_{1\alpha}$ (246 ± 88 pg mg^{-1}) and TXB$_2$ (3.4 ± 1 pg mg^{-1}) were not different from eicosanoid levels in vehicle-treated rats (116 ± 26, 208 ± 42 and 4.2 ± 0.5 pg mg^{-1}, respectively, $n = 6$ each group). Likewise, levels of PGE$_2$ and 6-keto-PGF$_{1\alpha}$ were not increased 5 min (123 ± 29 and 238 ± 43 pg mg^{-1}, respectively) or 10 min (109 ± 35 and 259 ± 68 pg mg^{-1}, respectively) after exposure of the mucosa to the mild irritant. Whereas indomethacin (20 mg kg^{-1}) near-maximally suppressed gastric mucosal levels of PGE$_2$ (8 ± 2 pg mg^{-1}), 6-keto-PGF$_{1\alpha}$ (12 ± 1 pg mg^{-1}) and TXB$_2$ (0.3 ± 0.02 pg mg^{-1}, $P < 0.001$ each vs rats treated with 20% ethanol alone, $n = 6$ per group), NS-398 (1 mg kg^{-1}) did not reduce mucosal levels of PGE$_2$ (129 ± 16 pg mg^{-1}), 6-keto-PGF$_{1\alpha}$ (292 ± 48 pg mg^{-1}) and TXB$_2$ (3.5 ± 0.8 pg mg^{-1}, $n = 6$ in all groups) 30 min after exposure to the mild irritant (Figures 5 and 6). Similarly, L-745,337 (2 mg kg^{-1}) and DFU (0.2 mg kg^{-1}) did not diminish

Table 1. Effect of indomethacin and NS-398 on the gastro-protection conferred by sodium salicylate, dimercaprol, indomethacin and lithium

Treatment	Vehicle	Pretreatment Indomethacin (20 mg kg^{-1})	NS-398 (1 mg kg^{-1})
Vehicle	32 ± 3	36 ± 4	29 ± 3
Sodium salicylate (100 mg kg^{-1})	$2 \pm 0.4*$	$3 \pm 0.5*$	$2 \pm 0.8*$
Dimercaprol (30 μg kg^{-1})	$3 \pm 0.5*$	$3 \pm 0.6*$	$1 \pm 0.4*$
Iodoacetamide (50 mg kg^{-1})	$1 \pm 0.5*$	$1 \pm 0.4*$	$1 \pm 0.3*$
Lithium (20 mg kg^{-1})	$2 \pm 0.6*$	$3 \pm 0.9*$	$1 \pm 0.7*$

Rats were treated orally with sodium salicylate, dimercaprol, iodoacetamide, lithium or vehicle 30 min before oral instillation of 1 ml of 70% ethanol. Additional groups of rats received indomethacin or NS-398 by gavage 30 min before administration of the protective agent. Results, shown as lesion index, are the mean $\pm$ s.e.mean of 4 experiments in each group. *$P < 0.001$ vs vehicle-treated control rats.

gastric mucosal levels of PGE$_2$ (123 ± 18 and 160 ± 26.3 pg mg^{-1}, respectively), 6-keto-PGF$_{1\alpha}$ (238 ± 68 and 259 ± 34 pg mg^{-1}, respectively) and TXB$_2$ (3.9 ± 0.4 and 4.3 ± 0.7 pg mg^{-1}, respectively) 30 min after instillation of 20% ethanol.

Gastric prostaglandins and thromboxane: mucosal synthesizing capacity Qualitatively identical results were found when the prostaglandin-synthesizing capacity of gastric mucosal fragments during a 10 min incubation at 37°C was assessed. Thus, 5, 10 or 30 min after treatment with vehicle, gastric mucosal
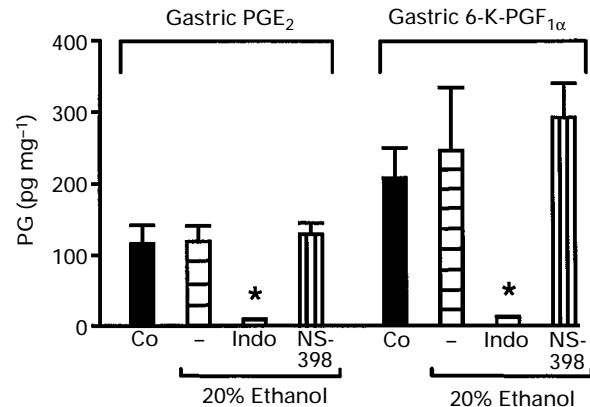
Figure 5 Effect of indomethacin and NS-398 on gastric mucosal prostaglandin levels. PGE$_2$ and 6-keto-PGF$_{1\alpha}$ were extracted from homogenized gastric mucosa obtained from rats 30 min after oral instillation of 1 ml of 20% ethanol and measured by RIA. Additional groups of rats were pretreated with indomethacin (Indo 20 mg kg^{-1}, p.o.) or NS-398 (1 mg kg^{-1}, p.o.) 30 min before instillation of 20% ethanol. Controls (Co) received the vehicle and 1 ml of H$_2$O instead of 20% ethanol. Each column represents the mean $\pm$ s.e.mean of 6 experiments. *$P < 0.001$ vs rats treated with vehicle 30 min before instillation of 20% ethanol.
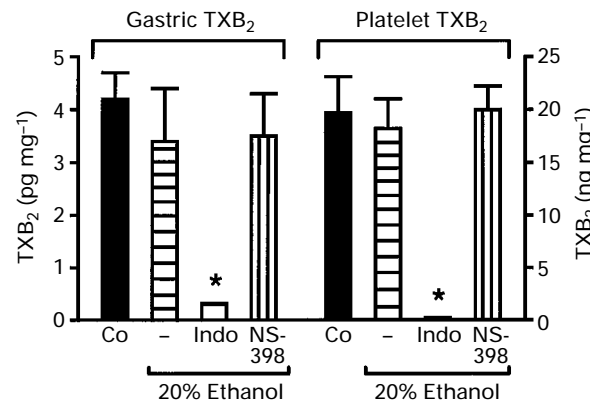
Figure 6 Effect of indomethacin and NS-398 on gastric mucosal TXB$_2$ levels and platelet TXB$_2$ formation. Gastric TXB$_2$ levels: TXB$_2$ was extracted from homogenized gastric mucosa obtained from rats 30 min after oral instillation of 1 ml of 20% ethanol and measured by RIA. Additional groups of rats were pretreated with indomethacin (Indo, 20 mg kg^{-1}, p.o.) or NS-398 (1 mg kg^{-1}, p.o.) 30 min before instillation of 20% ethanol. Controls (Co) received the vehicle and 1 ml of H$_2$O instead of 20% ethanol. Platelet TXB$_2$ formation: whole blood (0.5 ml) was obtained by cardiac puncture 30 min after oral instillation of 1 ml of 20% ethanol and was incubated at 37°C for 60 min. After centrifugation, serum TXB$_2$ levels were determined by RIA. Additional groups of rats were pretreated with indomethacin (20 mg kg^{-1}, p.o.) or NS-398 (1 mg kg^{-1}, p.o.) 30 min before instillation of 20% ethanol. Controls received the vehicle and 1 ml of H$_2$O instead of 20% ethanol. Each column represents the mean $\pm$ s.e.mean of 6 experiments. *$P < 0.001$ vs rats treated with vehicle before 20% ethanol.

fragments released comparable amounts of PGE_2 (810 ± 107, 789 ± 78 and 816 ± 85 pg mg^{-1} 10 min^{-1}, respectively) and 6-keto-$PGF_{1\alpha}$ (1012 ± 150, 979 ± 136 and 1001 ± 112 pg mg^{-1} 10 min^{-1}, respectively, $n=6$ each group). Five, ten or thirty minutes after treatment with 20% ethanol, mucosal release of PGE_2 (790 ± 110, 782 ± 97 and 830 ± 102 pg mg^{-1} 10 min^{-1}, respectively), and 6-keto-$PGF_{1\alpha}$ (990 ± 147, 1004 ± 120 and 994 ± 65 pg mg^{-1} 10 min^{-1}, respectively, $n=6$ each group) was not significantly different from that in vehicle-treated rats. Mucosal release of TXB_2 was 34 ± 3 pg mg^{-1} 10 min^{-1} 30 min after treatment with vehicle and 29 ± 4 pg mg^{-1} 10 min^{-1} 30 min after treatment with 20% ethanol ($n=6$ each group). Pretreatment with indomethacin (20 mg kg^{-1}) near maximally inhibited the synthesizing capacity of the mucosa for PGE_2 (42 ± 9 pg mg^{-1} 10 min^{-1}), 6-keto-$PGF_{1\alpha}$ (37 ± 6 pg mg^{-1} 10 min^{-1}) and TXB_2 (2 ± 0.1 pg mg^{-1} 10 min^{-1}) 30 min after instillation of the mild irritant. In contrast, treatment with NS-398 (1 mg kg^{-1}), L-745,337 (2 mg kg^{-1}) and DFU (0.2 mg kg^{-1}) was without effect on gastric mucosal release of PGE_2 (812 ± 87, 801 ± 92 and 831 ± 96 pg mg^{-1} 10 min^{-1}, respectively), 6-keto-$PGF_{1\alpha}$ (988 ± 85, 1012 ± 100 and 969 ± 93 pg mg^{-1} 10 min^{-1}, respectively) and TXB_2 (32 ± 4, 29 ± 3 and 31 ± 4 pg mg^{-1} 10 min^{-1}, respectively).

Platelet thromboxane formation Platelets generated 19.7 ± 3.4 ng ml^{-1} 60 min^{-1} TXB_2 in vehicle-treated control rats and 18.2 ± 2.8 ng ml^{-2} 60 min^{-1} in rats treated orally with 20% ethanol. Pretreatment with indomethacin (20 mg kg^{-1}) abolished the release of TXB_2 from platelets in rats treated with 20% ethanol. Pretreatment with NS-398 (1 mg kg^{-1}) was without effect (20 ± 2.2 ng ml^{-1} 60 min^{-1}) (Figure 6). Likewise, L-745,337 (2 mg kg^{-1}) and DFU (0.2 mg kg^{-1}) did not inhibit TXB_2 formation in platelets (17.1 ± 3.1 and 17.5 ± 3.5 ng ml^{-1} 60 min^{-1}, respectively, $n=6$ each group).

Effect of dexamethasone and NS-398 on release of PGE_2 into the inflammatory exudate

Five hours after implantation, the exudates accumulated in the carrageenin-soaked sponges in vehicle-treated control rats contained large amounts of PGE_2 (3.4 ± 0.5 ng ml^{-1}, $n=12$). Treatment with dexamethasone (3 mg kg^{-1}, administered p.o. 10 min before sponge implantation) reduced PGE_2 concentrations in the exudates to 0.5 ± 0.05 ng ml^{-1} ($P<0.001$, $n=5$).
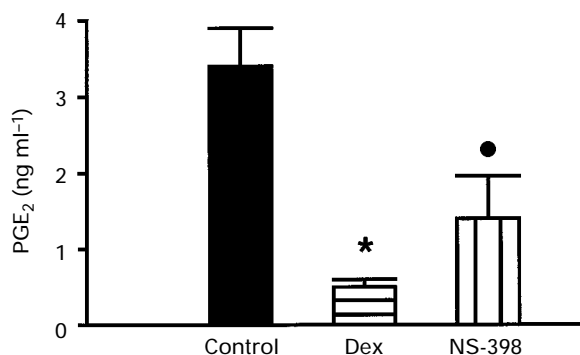

Figure 7 Effect of dexamethasone (Dex) and NS-398 on release of PGE_2 into inflammatory exudates. Rats were pretreated with dexamethasone (3 mg kg^{-1}, p.o., 10 min before) and NS-398 (1 mg kg^{-1}, p.o., 30 min before and 4 h after) implantation of carrageenin-soaked sponges. Concentrations of PGE_2 in the inflammatory exudates were measured 5 h after sponge implantation. Columns represent the mean$\pm$s.e.mean of 4–6 experiments. ●$P<0.05$; *$P<0.001$ vs controls.

Treatment with NS-398 (1 mg kg^{-1}, administered p.o. 30 min before and 4 h after sponge implantation) reduced PGE_2 concentrations in the exudates to 1.4 ± 0.56 ng ml^{-1} ($P<0.05$, $n=5$). Results are shown in Figure 7. Results published elsewhere (Schmassmann *et al.*, in press) have revealed that L-745,337 (2 mg kg^{-1}) inhibited inflammatory PGE_2 formation by 60%.

Discussion

Exogenous prostaglandins protect the gastric mucosa against a variety of noxious agents such as strong irritants, non-steroidal anti-inflammatory drugs, hot water, and other agents (Robert, 1979). Pretreatment of the gastric mucosa with mild irritants such as low concentrations of ethanol prevents gastric mucosal injury caused by strong irritants, a phenomenon that has been described as adaptive cytoprotection (Robert *et al.*, 1983). Since adaptive cytoprotection was reversed by indomethacin, it has been proposed that it is mediated through enhanced formation of endogenous prostaglandins in the gastric mucosa (Robert *et al.*, 1983). The results of our study confirm this observation. Thus, indomethacin inhibited the protective effect of the mild irritant 20% ethanol against mucosal damage caused by 70% ethanol in a dose-dependent manner (ID$_{50}$: 3.5 mg kg^{-1}). In addition, the study shows that identical reversal of protection conferred by 20% ethanol is brought about by pretreatment with the selective COX-2 inhibitors NS-398 (ID$_{50}$: 0.3 mg kg^{-1}), L-745,337 (ID$_{50}$: 0.4 mg kg^{-1}) and DFU (ID$_{50}$: 0.06 mg kg^{-1}). The highest doses of the non-selective COX inhibitor and the selective COX-2 inhibitors used abolished the protective effect of the mild irritant. Similar attenuation of the protective effect of 20% ethanol was observed when 96% ethanol was used to damage the mucosa.

The finding that selective COX-2 inhibitors have potent anti-inflammatory activity but do not cause gastric lesions (Arai *et al.*, 1993; Masferrer *et al.*, 1994; Chan *et al.*, 1995; Riendeau *et al.*, 1997; Schmassmann *et al.*, in press) initiated the idea that only COX-1 ensures the maintenance of mucosal integrity in the presence of noxious agents (Arai *et al.*, 1993; Masferrer *et al.*, 1994; Chan *et al.*, 1995; Vane & Botting, 1995; Riendeau *et al.*, 1997). Our results clearly show that NS-398, L-745,337 and DFU interfere with the protective effect of a mild irritant. These findings suggest that in certain circumstances COX-2-derived prostaglandins are involved in gastric mucosal defence reactions by increasing the resistance of the gastric mucosa in the face of pending injury.

Glucocorticoids prevent the induction of COX-2 and thus suppress the enhanced COX-2-mediated prostaglandin formation that occurs after an appropriate stimulus (Smith *et al.*, 1991; O'Banion *et al.*, 1992; O'Sullivan *et al.*, 1993; Mitchell *et al.*, 1995). In our study, pretreatment with dexamethasone did not inhibit the protection conferred by the mild irritant suggesting that it does not rely on an inducible COX-2. This hypothesis is supported by the finding that NS-398-sensitive protective effects of 20% ethanol could be demonstrated as early as 15 min after instillation of the mild irritant. Dexamethasone near-maximally inhibited release of PGE_2 into inflammatory exudates induced by subcutaneous implantation of carrageenin-soaked sponges, indicating that the compound at the dose used in the protection experiments has the potency to suppress expression of COX-2. Dexamethasone given in the absence of 20% ethanol, did not affect gastric mucosal damage caused by 70% ethanol excluding a protective effect of its own.

Oral instillation of 20% ethanol did not increase levels of PGE_2, 6-keto-$PGF_{1\alpha}$ and TXB_2 in the gastric mucosa. Likewise, the eicosanoid-synthesizing capacity of mucosal fragments as determined during an *ex vivo* incubation was not different in vehicle-treated and mild irritant-treated rats. Indomethacin at a dose that blocked the protective activity of the mild irritant abolished gastric mucosal formation of all eicosanoids as well as release of TXB_2 from platelets during clotting of whole blood. In contrast, NS-398, L-745,337 and DFU at doses that completely reversed the protective effect of 20% ethanol did not cause a measurable reduction of the levels of PGE_2, 6-keto-$PGF_{1\alpha}$ and TXB_2 and of the eicosanoid-synthesizing capacity in the gastric mucosa. Furthermore, the selective COX-2 inhibitors NS-398, L-745,337 and DFU did not reduce the release of TXB_2 from platelets. These findings are in keeping with previous data showing that selective COX-2 inhibitors do not affect gastric mucosal prostaglandin formation under basal conditions (Masferrer *et al.*, 1994; Chan *et al.*, 1995; Kishimoto *et al.*, 1997; Schmassmann *et al.*, in press) and clearly indicate that the reversal of mild irritant-evoked protection induced by NS-398, L-745,337 and DFU cannot be attributed to inhibition of COX-1.

Our finding that exposure of the gastric mucosa to 20% ethanol does not result in a measurable increase in prostaglandin formation is in contrast to the initial results by Robert *et al.*, (1983) who showed that the mild irritant NaOH, administered in protective concentrations, markedly increased gastric mucosal prostaglandin formation *ex vivo*. The reason for this discrepancy is not obvious so far. The lack of measurable stimulation of gastric mucosal prostaglandin formation by the mild irritant observed in our experiments may explain why inhibition of mucosal eicosanoid formation by the selective COX-2 inhibitors could not be demonstrated. In the gastric mucosa, COX-2-derived prostaglandins may represent only a minority of the prostaglandin pool. In this tissue, the large amounts of prostaglandins generated via the COX-1 isoform may create a background that renders isolated assessment of changes of COX-2 activity difficult. In contrast, both NS-398 and L-745,337 at doses that counteracted the protective effect of 20% ethanol significantly inhibited PGE_2 levels in inflammatory exudates confirming previous findings (Masferrer *et al.*, 1994; Chan *et al.*, 1995; Schmassmann *et al.*, in press).

NS-398 did not prevent the protection conferred by sodium salicylate, dimercaprol, iodoacetamide and lithium. The mechanisms underlying protection by these agents are not fully understood but do not involve the endogenous prostaglandin system as the protective effects were insensitive to reversal by the non-selective COX inhibitor indomethacin. The observation that the selective COX-2 inhibitors did not modify prostaglandin-independent types of protection, and did not influence gastric mucosal damage caused by 70% ethanol in the absence of the mild irritant, excludes nonspecific injury-modifying effects. Furthermore, pretreatment with 16,16-dimethyl-PGE_2 at 4 ng kg^{-1} fully reversed the inhibitory effects of NS-398, L-745,337 and DFU on the protection conferred by 20% ethanol. This dose of the PGE_2 analogue did not protect against mucosal damage induced by 70% ethanol when given alone. Obviously, doses of the PGE_2 analogue necessary to replace endogenous prostaglandins depleted after treatment with selective COX-2 inhibitors are lower than those that confer protection as a pharmacological effect, when endogenous prostaglandin production is not suppressed. The prostaglandin analogue at 4 ng kg^{-1} did not reverse the inhibitory effect of indomethacin. Using a non-ethanol damage model of experimental ulceration we could show that 16,16-

dimethyl PGE_2 at a dose of 10 ng kg^{-1} counteracts the interference with mucosal resistance elicited by indomethacin (unpublished observations). However, as 16,16-dimethyl PGE_2 has particularly potent protective activity against ethanol-evoked mucosal injury and, when given alone, at doses >4 ng kg^{-1} caused full protection in the ethanol damage model, the effect of prostaglandin replacement therapy on indomethacin-induced attenuation of the protective action of the mild irritant could not be revealed. The reversal of the effects of selective COX-2 inhibitors by prostaglandin administration supports the proposal that mild irritant-evoked protection occurs through COX-2-derived prostaglandins. However, we cannot completely exclude the possibility that effects other than suppression of prostaglandin production mediate the attenuation of mild irritant-induced protection elicited by the selective COX-2 inhibitors studied. So far, pharmacological effects of selective COX-2 inhibitors have been attributed exclusively to suppression of COX-2-mediated prostaglandin formation. If these compounds have additional influences on biological systems not related to COX-2, the mechanisms underlying their actions have to be re-evaluated.

Constitutively expressed levels of COX-2, in addition to COX-1, are present in normal unstimulated gastric tissues of various species. Thus, the occurrence of COX-2 mRNA and COX-2 protein in small but possibly strategic areas in normal rat gastric mucosa with particularly strong expression in the endothelia of gastric mucosal microvessels has recently been described (Tarnawski *et al.*, 1996). In another study, immunoreactivity for COX-2 could be localized in surface mucus cells in the fundic and pyloric region of the unstimulated rat stomach (Iseki, 1995). By use of immunoblot analysis, expression of low but detectable levels of COX-2 protein could be demonstrated in microsomes prepared from gastrointestinal tissues of normal rats (Kargman *et al.*, 1996). In the human stomach, approximately equivalent levels of COX-1 and COX-2 mRNA were present when reverse transcriptase-polymerase chain reaction was applied to quantify the COX isoforms (O'Neill & Ford-Hutchinson, 1993). In normal human gastric mucosa, endothelial cells of the microvessels, basement membranes and mucosal macrophages demonstrated strong expression of COX-2 mRNA and protein (Tarnawski *et al.*, 1997). Furthermore, immunostaining experiments revealed expression of COX-2 in myofibroblasts and endothelial cells of normal human gastric mucosa and showed that COX-2 was expressed constitutively in human gastric endothelial cells maintained in culture (Donnelly *et al.*, 1997).

In rat gastric mucosa, markedly elevated levels of COX-2 mRNA were observed after induction of damage by ischaemia-reperfusion (Kishimoto *et al.*, 1997). Furthermore, in mice, COX-2 mRNA and COX-2 protein levels markedly increased during ethanol-induced acute mucosal injury and in chronic gastric ulcers produced by subserosal injection of acetic acid (Mizuno *et al.*, 1997). We and others have recently shown that treatment with selective COX-2 inhibitors significantly delays healing in chronic experimental ulcers in rats and mice (Mizuno *et al.*, 1997; Schmassmann *et al.*, in press). The delay in healing caused by selective COX-2 inhibitors was comparable to that evoked by treatment with the conventional non-steroidal anti-inflammatory drugs indomethacin and diclofenac (Schmassmann *et al.*, in press). Formation of prostaglandins was found to be increased in the vicinity of experimental chronic gastric ulcers and was sensitive to *in vitro* inhibition by NS-398, whereas basal gastric mucosal prostaglandin formation was not affected (Mizuno *et al.*, 1997). These findings show that induction of COX-2 occurs in the

gastric mucosa during ulceration, possibly as a consequence of the accompanying inflammatory reaction, and suggest that COX-2-derived prostaglandins are essential to ensure rapid healing. In the rat stomach, chronic endotoxin treatment significantly increased expression of mRNA and protein for both COX-1 and COX-2 and resulted in increased resistance of the mucosa against injury through a COX-1- but not COX-2-dependent pathway (Ferraz *et al.*, 1997). Taken together, these observations suggest that regarding COX-1 as 'constitutive' and COX-2 as 'inducible' forms of prostaglandin synthase may be an oversimplification, since both constitutive and inducible COX-1 and COX-2 exist in the gastric mucosa and contribute to preservation of mucosal integrity in certain circumstances.

In conclusion, the present study shows that selective inhibitors of COX-2, although they do not produce gastric mucosal lesions, abolish the protective activity of a mild irritant, suggesting that COX-2-derived prostaglandins play a role in gastric mucosal defence reactions. The COX-2-mediated protection occurs rapidly, within 15 min, and is not affected by pretreatment with dexamethasone, indicating that enzyme induction is not involved. These findings provide the first evidence that COX-2-derived prostaglandins not only participate in pathophysiological events of the gastrointestinal tract, such as inflammation, ulcer healing or tumour growth, but that prostaglandins biosynthesized by a constitutive COX-2 enzyme may be essential mediators in physiological functions that are involved in homeostasis reactions in the stomach.

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