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# *Aspirin causes rapid up-regulation of cyclo-oxygenase-2 expression in the stomach of rats*

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## SUMMARY

*Background*: Cyclo-oxygenase-1 (COX-1) is believed to produce prostaglandins vital to mucosal defence, whereas cyclo-oxygenase-2 (COX-2) is induced at sites of inflammation. Little is known about the regulation of COX-2 in the stomach, particularly during the period following mucosal injury. In this study, we examined COX-1 and COX-2 expression shortly after administration of NSAIDs or ethanol.
*Methods*: Fasted rats were given aspirin, salicylate, indomethacin or ethanol (20% or 40%) orally. Three hours later the stomach was excised, the severity of damage scored and samples taken for RT-PCR of COX-1 and COX-2 mRNA and immunohistochemistry. Nitric oxide synthase mRNA (iNOS and eNOS) and activity were also measured.
*Results*: Aspirin, indomethacin and the higher concentration of ethanol produced widespread mucosal damage, whereas salicylate and 20% ethanol caused only superficial epithelial damage. Aspirin caused a significant increase in COX-2 mRNA expression and a marked increase in COX-2 immunoreactivity, particularly in the superficial mucosa. Expression of COX-1 (mRNA and protein) was unaffected by aspirin, as were NOS mRNA expression and enzyme activity. Pre-treatment with prostaglandin $E_2$ prevented the induction of COX-2 by aspirin. Salicylate and indomethacin caused modest increases in COX-2 immunoreactivity but no change in COX-2 mRNA. Neither concentration of ethanol affected COX-2 mRNA or protein expression, suggesting that this was a specific response to the aspirin, rather than to injury.
*Conclusions*: These results demonstrate a rapid up-regulation of COX-2 expression in response to aspirin, possibly representing a compensatory response to inhibition of gastric prostaglandin synthesis.

## INTRODUCTION

Prostaglandins and nitric oxide (NO) have the capacity to influence many components of what is collectively termed 'gastric mucosal defence'.[1] The importance of these mediators is exemplified by the fact that suppression of mucosal prostaglandin or NO synthesis renders the gastrointestinal tract more susceptible to injury.[2, 3] In recent years, it has been clearly demonstrated that enzymes responsible for the synthesis of prostaglandins and NO exist in multiple isoforms. Two forms of the cyclo-oxygenase (COX) enzyme have been characterized. The predominant isoform of COX in a normal stomach is COX-1,[4] and its expression can increase in some circumstances.[5] Conversely, COX-2 is not constitutively expressed in most tissues but can be induced by exposure to certain cytokines, mitogens and endotoxin, and is expressed at sites of inflammation, including in the gastrointestinal tract.[5–7] Nitric oxide synthase (NOS) is also constitutively expressed in the stomach, most notably in the endothelium (eNOS) and in enteric neurons (bNOS).[8] Inducible NOS (iNOS) can

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be found expressed in the stomach following administration of endotoxin,[9] and the NO produced from this isoform can exert protective effects in the stomach.[10]

The discovery of two distinct isoforms of COX has led to the proposal that selective COX-2 inhibitors will reduce inflammation while sparing the gastrointestinal tract from damage.[11–13] However, it has been suggested that, in situations in which the mucosa is inflamed, COX-2 is likely to be expressed and produce prostaglandins that contribute to mucosal defence and ulcer healing.[2, 6, 7] For example, in an animal model of colitis, administration of selective inhibitors of COX-2 resulted in exacerbation of tissue injury,[6] while in a mouse model of gastric ulcer, administration of a selective COX-2 inhibitor delayed healing.[7] Little is known of the regulation of COX-2 expression within the gastrointestinal tract following acute injury.

In this study, we examined the effects of administration of agents that produce mucosal injury on the expression of COX-2 (mRNA and protein). In particular, we examined the changes in COX-2 expression shortly after administration of NSAIDs or ethanol.

## METHODS

### *Animals*

Male Wistar rats weighing 175–200 g were used in all experiments. The rats were obtained from Charles River Breeding Farms (Montréal, Québec) and were housed in polypropylene cages and fed standard laboratory chow and tap water *ad libitum*. All experimental procedures were approved by the Animal Care Committee of the University of Calgary and were in accordance with the guidelines of the Canadian Council on Animal Care.

### *Acute gastric damage*

Rats ($n = 6$ per group) were fasted overnight then given aspirin (250 mg/kg), sodium salicylate (281 mg/kg), indomethacin (20 mg/kg), 20% or 40% ethanol, or vehicle (1% carboxymethylcellulose) orally. In each case, the total volume administered was 0.5 mL. The dose of sodium salicylate used was selected because it is equimolar to the dose of aspirin. Three hours later the rats were killed by cervical dislocation and the stomach was excised and opened by an incision along the greater curvature. The assessment of macroscopic damage was performed by an observer unaware of the treatment the rats had received, as described previously.[14] Briefly, this method involved measuring the length of each lesion in millimetres, then summing the lengths of all lesions observed in each stomach.

### *Quantification of mRNA expression*

Gastric COX-1, COX-2 and nitric oxide synthase (eNOS and iNOS) mRNA expression were examined using the reverse transcriptase polymerase chain reaction (RT-PCR) technique. Samples of the stomach (corpus) were taken from rats that were treated as described above and were immediately frozen in a 50% (w/v) guanidinium solution containing 26.4 mmol sodium citrate (pH 7.0), 0.528% sarcosyl and 0.0072% $\beta$-mercaptoethanol. For each 100 mg of tissue, 1 mL of the guanidinium solution was used. Total RNA was isolated using the acid guanidinium isothiocyanate method, as described previously.[15]

The method used for RT-PCR was modified slightly from that described previously.[16] The housekeeping gene for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Briefly, 1 $\mu$g of RNA from each sample was reverse transcribed at 42 °C using Superscript RNase H Reverse Transcriptase Gibco BRL (Gaithersburg, MD, USA) and the appropriate reaction mixture (containing 2 $\mu$L 10X PCR buffer, 2 $\mu$L 10 mmol dNTP stock and 2 $\mu$L $N_6$ random hexamer stock). The enzyme was then deactivated by heating the samples to 95 °C for 10 min. After the reaction, 2 $\mu$L of cDNA was mixed with 2 $\mu$L 2 mmol dNTP stock and 2 $\mu$L 10X PCR buffer. Two microlitres of the upstream primer (≈1 pmol) and 2 $\mu$L of the downstream primer (≈1 pmol) under investigation were then added to each tube.

DNA amplification was carried out under the following conditions: denaturation at 94 °C for 1 min, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. To ensure complete denaturation of the DNA with no background polymerase activity, Taq DNA polymerase was added to the PCR mixture during the hot start of cycle 1. Optimal co-amplification of each primer with GAPDH was determined during preliminary trials (data not shown). The COX-1/GAPDH genes were co-amplified for 30/22 cycles, whereas the COX-2/GAPDH, eNOS/GAPDH and iNOS/GAPDH genes were co-amplified for 33/23, 29/22 and 30/23 cycles, respectively. Hence, the GAPDH upstream and downstream primers were added to the COX-1/COX-2/eNOS/iNOS PCR

mixture during the hot start of cycle 9, 11, 8 and 8, respectively.

After separation of the PCR products on a 2% agarose gel containing 10 $\mu$g ethidium bromide, a Polaroid picture of the gel was taken under ultraviolet light. Using a densitometer and National Institutes of Health software (Image), quantities of each product were normalized to control levels of GAPDH and expressed as densitometry units. The COX-1, COX-2, eNOS, iNOS and GAPDH RT-PCR products were made using primers based on published sequences (Table 1).[17–23]

*COX immunohistochemistry*

Tissues from control rats and rats treated with acute aspirin, sodium salicylate, indomethacin and ethanol (20% or 40%) ($n = 4$–10 per group) were harvested 3 h post-treatment and fixed by immersion in Zamboni's fixative overnight at 4 °C. After fixation, they were washed in phosphate-buffered saline (PBS, pH 7.4) and processed for indirect immunofluorescence as cryostat sections (12 $\mu$m). Sections were incubated in primary antibodies for 48 h at 4 °C. Primary antibodies raised in rabbit against a peptide sequence from the human COX-1 or COX-2[24] were used (1:500). Previous studies have demonstrated the specificity of these antibodies.[6] Tissues were viewed with an epifluoresence microscope (Axioplan; Carl Zeiss Inc., Thornwood, NY) and photographed using TMax 400 ASA black and white film (Eastman Kodak Co., Rochester, NY).

*Nitric oxide synthase activity*

NOS activity was determined as the conversion of radiolabelled L-arginine to citrulline by the methods described previously.[9] Segments of the corpus (≈200 mg) were removed from rats killed via cervical dislocation 3 h after oral administration of 250 mg/kg aspirin, or 0.5 mL of 40% ethanol or vehicle, and stored at −70 °C for 7 days prior to analysis. Tissues were homogenized (30 s) using a Polytron homogenizer in 250 $\mu$L ice-cold buffer (HEPES 10 nmol, sucrose 320 nmol, dithiothreitol 1 nmol, EDTA 0.1 nmol, soybean trypsin inhibitor 0.01 mg/mL, leupeptin 0.01 mg/mL and aprotinin 0.002 mg/mL) adjusted to pH 7.4 (8 mol NaOH) followed by centrifugation for 10 min at 14 000 ***g*** at 4 °C. A 20 $\mu$L sample of supernatant was incubated for 10 min at 37 °C in 50 $\mu$L of NOS reaction buffer comprising (final concentrations): $KH_2PO_4$ 50 mmol, $MgCl_2$ 1 mmol, $CaCl_2$ 0.2 mmol, valine 50 mmol, NADPH 0.3 mmol, L-arginine 3.15 mg/mL and [$^{14}$C]-L-arginine 157 pmol (110 000 d.p.m./mL). The reaction was incubated in a 37 °C shaking water bath for 10 min. The reaction was arrested via the removal of the substrate. This was achieved by the addition of 0.5 mL of a 1:1 suspension of Dowex (AG 50 W-X8) : water, followed by vortex mixing. The mixture was run into Dowex columns and rinsed with 2 mL of deionized water. Unreacted L-arginine binds to the resin, while L-citrulline is eluted. The eluent was collected and a 1 mL aliquot was added

Table 1. Primer sequences for RT-PCR

| Gene | Primer sequences | Amplicon length (base pairs) | Reference(s) |
|---|---|---|---|
| COX-1 | | | |
| Upstream | 5′-CCT TCT CCA ACG TGA GCT ACT A-3′ | | |
| Downstream | 5′-TCC TTC TCT CCT GTG AAC TCC T-3′ | 1036 | 17 |
| COX-2 | | | |
| Upstream | 5′-AGA CAG ATC ATA AGC GAG GAC C-3′ | | |
| Downstream | 5′-CAC TTG CAT TGA TGG TGG CTG T-3′ | 1158 | 18–20 |
| eNOS | | | |
| Upstream | 5′-GGA GAA GAT GCC AAG GCT GCT G-3′ | | |
| Downstream | 5′-CTT CCA GTG TCC AGA CGC ACC A-3′ | 224 | 21 |
| iNOS | | | |
| Upstream | 5′-ACA ACA GGA ACC TAC CAG CTC A-3′ | | |
| Downstream | 5′-GAT GTT GTA GCG CTG TGT GTC A-3′ | 651 | 22 |
| GAPDH | | | |
| Upstream | 5′-CGG AGT CAAC GGA TTT GGT CGT AT-3′ | | |
| Downstream | 5′-AGC CTT CTC CAT GGT GGT GAA GAC-3′ | 306 | 23 |

to 14 mL scintillation fluid for estimation of the radiolabelled products by $^{14}C$ scintillation counting.

NOS activity was defined as the rate of citrulline formation and was further characterized by the effects of incubation *in vitro* with EGTA (1 mmol). Thus, basal NOS activity that was abolished by EGTA was taken as calcium-dependent constitutive NOS, whereas that not inhibited by EGTA incubation was taken as calcium-independent NOS activity.

### *Statistical analysis*

All data are expressed as the mean ± S.E.M. Comparisons among groups of data were made using one-way analysis of variance followed by a Student–Newman–Keuls test. With all analyses, an associated probability ($P$-value) of less than 5% was considered significant.

### *Materials*

Sarcosyl and isopropanol were obtained from Sigma Chemical Company (St. Louis, MO). L-[U-$^{14}C$]-arginine monohydrochloride was obtained from NEN-DuPont (Oakville, ON, Canada). $\beta$-mercaptoethanol was obtained from Bio Rad Laboratories Inc. (Mississauga, ON, Canada). PCR buffer and dNTP stock were obtained from Pharmacia Biotech Inc. (Mississauga, ON, Canada). Sodium citrate, phenol and superscript RNase H reverse transcriptase were obtained from Gibco BRL (Gaithersburg, MD). COX-1 and GADPH-primers were synthesized by University Core DNA Services (University of Calgary, Calgary, AB, Canada). The antibodies directed against COX-1 and -2 were generously provided by Dr Chan and Dr I. Rodger of Merck-Frosst Therapeutic Research Centre (Montréal, QC, Canada).

## RESULTS

### *Acute gastric damage*

Oral administration of aspirin, 40% ethanol or indomethacin caused extensive haemorrhagic damage in the corpus region of the stomach (Figure 1). The lesions were usually linear and confined to the corpus region, often located on the crests of the rugal folds. In contrast to these findings, and consistent with previous reports,[25, 26] the gastric mucosa of rats exposed to salicylic acid and 20% ethanol exhibited only superficial epithelial sloughing, with only a few haemorrhagic erosions, and the mean gastric damage scores in these groups did not differ significantly from that in rats treated only with vehicle (Figure 1).

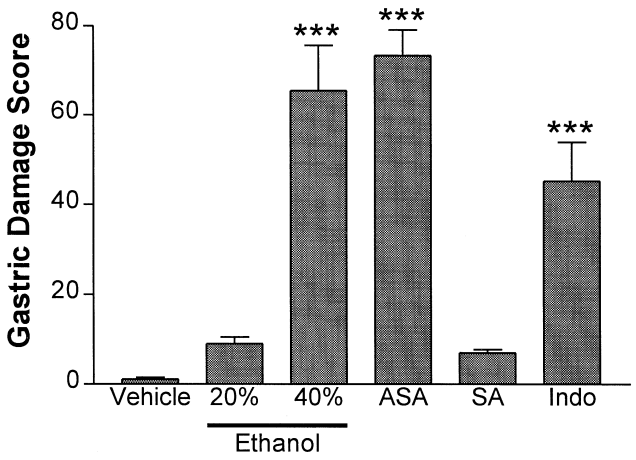


Figure 1. Extent of gastric damage observed 3 h after oral administration of aspirin (ASA 250 mg/kg), indomethacin (20 mg/kg), sodium salicylate (SA 281 mg/kg) or ethanol (20% or 40%). ***$P < 0.001$ compared to the vehicle-treated group.

### *COX mRNA expression*

Oral administration of aspirin at 250 mg/kg, a dose which inhibits systemic COX activity by >99%,[27] significantly increased the expression of COX-2 mRNA in gastric tissues (Figure 2). However, neither of the ethanol concentrations tested, nor indomethacin or

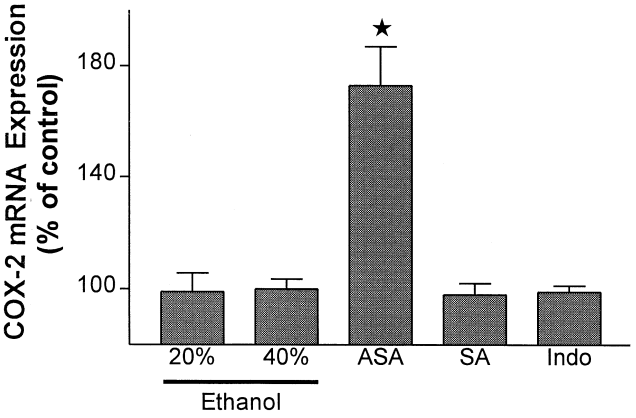


Figure 2. Gastric expression of mRNA for COX-2, 3 h after oral administration of aspirin (250 mg/kg), indomethacin (20 mg/kg), sodium salicylate (281 mg/kg) or ethanol (20% or 40%). The results are expressed as a percentage of the expression observed in vehicle-treated rats. Each group consisted of 4–10 rats. *$P < 0.05$ compared to the vehicle-treated group.

salicylate, significantly affected gastric COX-2 mRNA expression. Aspirin did not alter gastric expression of COX-1 mRNA (78 ± 4% of control levels, $n = 6$ per group).

### *COX immunohistochemistry*

In tissues from control rats, COX-1 immunoreactivity was detectable and no significant changes were observed after aspirin treatment (data not shown). COX-2 immunoreactivity was not detectable in tissues from control rats (Figure 3A). In gastric tissue from rats treated with either 20% or 40% ethanol, COX-2 immunoreactivity was not observed (Figure 3B). In rats treated with aspirin, indomethacin or salicylic acid, there was diffuse staining for COX-2 in the mucosa. The most intense COX-2 immunoreactivity was evident after aspirin administration (Figure 3C), and was localized to the superficial mucosa. The nature of the cells staining for COX-2 is not yet clear.

Oral administration of prostaglandin $E_2$ prior to aspirin treatment resulted in a reduction in the gastric damage score from 73.4 ± 5.8 to 0.8 ± 0.5 ($P < 0.001$) and was accompanied by marked preservation of mucosal architecture. COX-2 expression in the gastric mucosa was still observed in this group, but it was substantially reduced from that observed in rats treated only with aspirin (Figure 3D). Expression of COX-2 mRNA in rats pretreated with $PGE_2$ did not differ significantly from that in control rats.

### *NOS mRNA expression and activity*

Aspirin administration did not significantly affect expression of iNOS or eNOS mRNA in the stomach (Table 2). $Ca^{2+}$-dependent and -independent NOS activity was detectable in homogenates of normal rat gastric mucosa (Table 2). Oral administration of aspirin 250 mg/kg did not significantly alter either form of NOS activity.

## DISCUSSION

Prostaglandins play a key role in modulating gastric mucosal defence, and also appear to be important for repair of mucosal injury.[1, 2] Indeed, prostaglandins have been shown to be important mediators of epithelial restitution in the stomach,[28, 29] and their importance in repair of ulcers is underscored by the marked ability of NSAIDs to interfere with this process.[30, 31] While it is now clear that there are at least two isoforms of cyclooxygenase (the principle enzyme responsible for prostaglandin synthesis), the relative contribution of each isoform to mucosal prostaglandin synthesis in various circumstances is not clear. In the normal stomach, only COX-1 is expressed in detectable amounts.[4, 5, 7] Following chronic endotoxin administration or induction of chronic gastric ulcers, COX-2 expression is pronounced.[5, 7] The results of the present study indicate that COX-2 can also be expressed very quickly after administration of aspirin, and possibly other NSAIDs. Aspirin caused a significant increase in COX-2 mRNA expression in the stomach, which was accompanied by a marked increase in immunohistochemically detectable COX-2 protein. Indomethacin (a potent inhibitor of COX-1 and COX-2) and salicylate (a weak inhibitor of both COX-1 and COX-2)[11] caused a modest increase in COX-2 protein expression in the stomach, but did not affect mRNA expression. The elevation in COX-2 expression in these circumstances was not a non-specific response to injury, because administration of ethanol at concentrations causing superficial (20% v/v) or deep (40% v/v) mucosal injury failed to significantly alter mRNA or protein expression. Moreover, the effects of aspirin were specific to COX-2, because no effect on COX-1, iNOS or eNOS expression were detected. Like COX-2, iNOS is a rapidly inducible enzyme that is expressed at sites of inflammation and injury in the gastrointestinal tract.[8]

The dose of aspirin used in this study is sufficient to cause profound suppression of systemic COX activity[27] and prostaglandin synthesis by the stomach.[32] It is therefore possible that the increase in COX-2 expression following aspirin administration occurred as a response to diminished tissue prostaglandin synthesis. While this remains unproven, the observation that oral administration of $PGE_2$ prior to aspirin resulted in inhibition of the up-regulation of COX-2 expression supports this hypothesis.

It is interesting that aspirin had a much more pronounced effect on immunohistochemically detectable COX-2 protein expression than indomethacin or salicylate, and was the only treatment of the three to significantly increase COX-2 mRNA expression in this study. This may be due to the fact that, unlike other NSAIDs, aspirin irreversibly inhibits COX activity by acetylating a serine residue within the active site of the enzyme.[33] Moreover, there is evidence that, in contrast

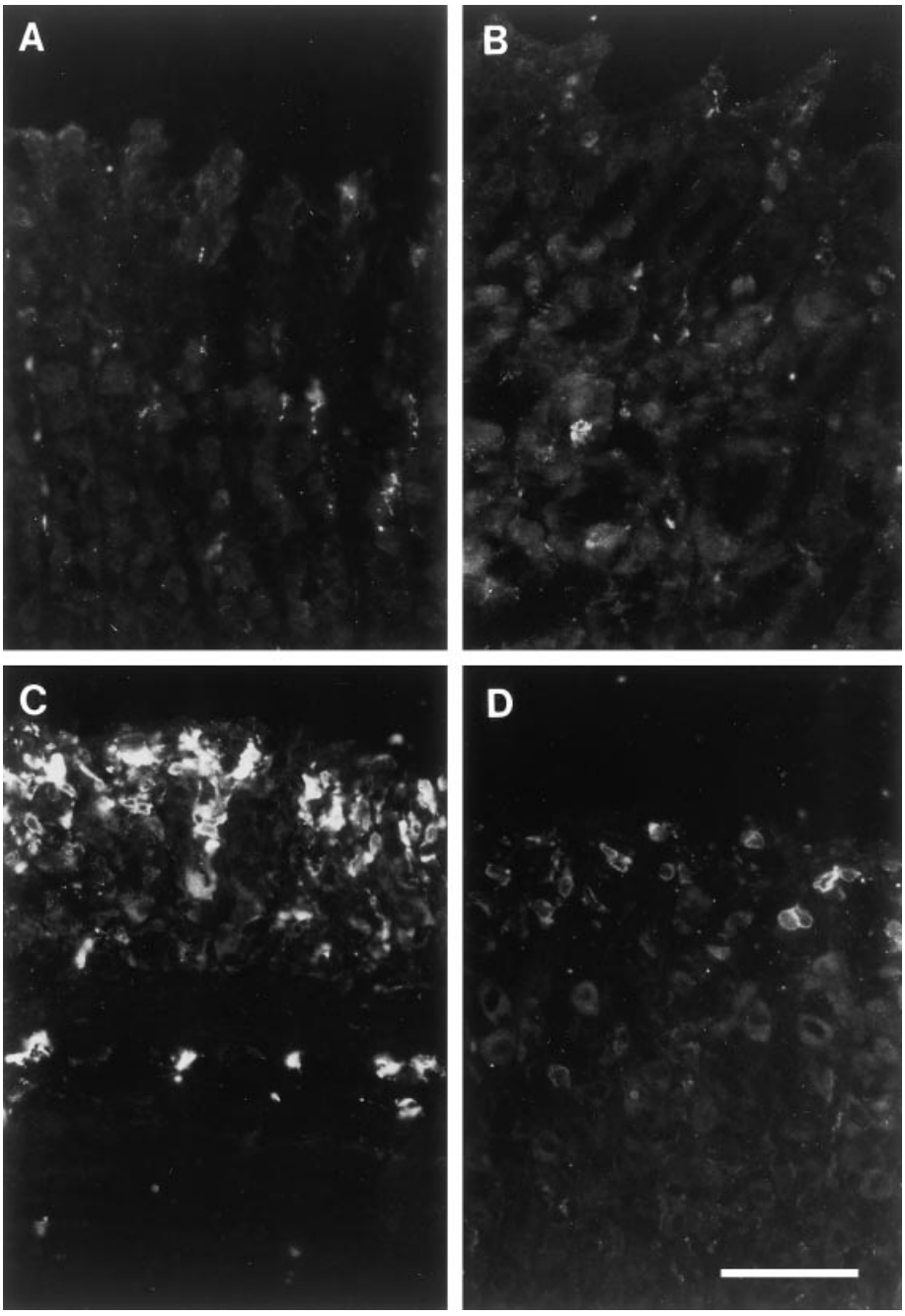


Figure 3. Fluorescence micrographs of COX-2 immunoreactivity in sections of stomach from normal (A) and treated (B–D) rats. COX-2 immunoreactivity was virtually absent in untreated control rats (A) and in rats treated with 20% ethanol (B), even though mucosal architecture was clearly disrupted in the latter group. In contrast, cellular COX-2 protein expression was markedly up-regulated in the damaged mucosa after treatment with aspirin (C). This up-regulation was to a large extent attenuated in rats receiving $PGE_2$ prior to aspirin (D), although some immunoreactive cells were still present in the mucosa. Scale bar 50 $\mu$m.

to its effects on COX-1, aspirin does not render COX-2 completely inactive. Although prostaglandin generation is inhibited, aspirin-acetylated COX-2 has been shown to produce 15-hydroxyeicosatetraenoic acid.[33] The functional significance of the 15-hydroxyeicosatetraenoic acid produced in this circumstance is not yet known.

COX activity was not measured in this study, because a number of the test drugs (e.g. aspirin and

Table 2. Effects of aspirin on gastric iNOS and eNOS mRNA expression and activity

| | Vehicle | Aspirin |
|---|---|---|
| iNOS mRNA expression (% of control) | 100 ± 5 | 119 ± 4 |
| eNOS mRNA expression (% of control) | 100 ± 5 | 113 ± 2 |
| iNOS activity (nmol/min/mg) | 0.09 ± 0.01 | 0.06 ± 0.03 |
| eNOS activity (nmol/min/mg) | 0.94 ± 0.06 | 0.60 ± 0.18 |

There were no significant differences between the vehicle- and aspirin-treated groups. ($n = 6$ per group for RT-PCR; $n = 4$–5 per group for NOS activity).

indomethacin) profoundly suppress activity of COX-1 and COX-2 at the doses used, however, previous studies have shown that induction of COX-2 in the gastrointestinal tract is accompanied by increased prostaglandin synthesis from that isoform. For example, Reuter and co-workers[6] documented increased COX-2-derived prostaglandin synthesis in the colon in association with increased COX-2 protein and mRNA expression, and Mizuno and co-workers[7] showed a similar relationship in the mouse stomach following induction of an ulcer.

In summary, administration of aspirin at a dose that caused profound suppression of systemic COX activity and extensive gastric mucosal damage resulted in a rapid induction of COX-2 expression in the stomach. This effect could be reversed by administration of $PGE_2$, which suggests that depressed mucosal prostaglandin synthesis may be the trigger for induction of COX-2. Moreover, the lack of induction of this enzyme by 20% or 40% ethanol, and the lack of induction of iNOS in response to aspirin, indicates that this effect was not a non-specific response to tissue injury.

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