

## BASIC-ALIMENTARY TRACT

# The Effects of Aspirin on Gastric Mucosal Integrity, Surface Hydrophobicity, and Prostaglandin Metabolism in Cyclooxygenase Knockout Mice

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See editorial on page 341.

**Background & Aims:** Insight into the role of the different cyclooxygenase isoforms in prostaglandin biosynthesis, surface hydrophobicity, and gastric mucosal barrier integrity can be gained by comparing the effects of luminal damaging agents in wild-type and cyclooxygenase knockout mice. **Methods:** Fasted wild-type, cyclooxygenase-1, and cyclooxygenase-2 knockout mice were intragastrically administered saline, 0.6N HCl, or aspirin (aspirin 20 mmol/L) in combination with 0.6N HCl and killed 1 hour later, at which time the gastric lesion score was assessed and biopsy samples were taken for surface, biochemical, and morphological analyses. **Results:** The gastric mucosa of cyclooxygenase-1 knockout mice was more severely injured by both HCl alone and aspirin/HCl than that of wild-type and cyclooxygenase-2 knockout mice. HCl alone and aspirin/HCl also induced a more profound decrease in surface hydrophobicity in cyclooxygenase-1 knockout mice than in wild-type mice, whereas this surface property was unaffected in cyclooxygenase-2 knockout mice. The gastric injury induced by aspirin/HCl in cyclooxygenase-1 knockout mice could be prevented if the animals were treated with phosphatidylcholine-associated aspirin. Aspirin/HCl, in comparison to saline or HCl alone, induced a 4–6-fold increase in gastric mucosal prostaglandin E<sub>2</sub> concentration in the cyclooxygenase-1 knockout mice, whereas it decreased prostaglandin E<sub>2</sub> levels in wild-type and cyclooxygenase-2 knockout mice. This paradoxical aspirin-induced increase in gastric prostaglandin E<sub>2</sub> in cyclooxygenase-1 knockout mice seemed to correspond to an increase in cyclooxygenase-2 messenger RNA and protein expression. The gastric lesion score seemed to be significantly associated with alterations in surface hydrophobicity but not with mucosal prostaglandin E<sub>2</sub> concentration. **Conclusions:** Our evidence on cyclooxygenase knockout mice suggests that aspirin predominantly causes gastric injury by a non-

prostaglandin mechanism, perhaps by attenuating surface hydrophobicity, a possibility supported by the low gastric toxicity of phosphatidylcholine/aspirin. However, prostaglandins generated by cyclooxygenase-1 may play an important permissive role in maintaining gastric mucosal barrier integrity. Aspirin seems to paradoxically increase the gastric mucosal prostaglandin E<sub>2</sub> concentration in cyclooxygenase-1 knockout mice, possibly by the induction of cyclooxygenase-2.

The role of prostaglandins in the mechanism of gastric mucosal defense has been intensively explored since Robert et al.<sup>1</sup> showed in rats that certain eicosanoids possessed a remarkable ability to increase the stomach's resistance to contrasting luminal damaging agents. These studies also provided important insight into why a class of nonsteroidal anti-inflammatory drugs (NSAIDs), which share in the ability to block prostaglandin biosynthesis by inhibiting the rate-limiting enzyme cyclooxygenase (COX), may induce injury to the upper gastrointestinal (GI) tract by depleting the tissue of cytoprotective prostaglandins.<sup>2</sup>

Because of the development of powerful molecular tools over the past decade, we now know that at least 2, and possibly 3 or more, related isoforms of COX are present. COX-1 is present predominantly and is constitutively expressed in platelets, epithelia of the GI tract, and other tissues (e.g., kidney, liver, and selected neural tissue). COX-2 is expressed primarily by cytokine induction at sites of inflammation.<sup>3–6</sup> Recent evidence has

*Abbreviations used in this paper:* bp, base pair; CLASS, Celecoxib Long-term Arthritis Safety Study; COX, cyclooxygenase; GI, gastrointestinal; PC, phosphatidylcholine; PCR, polymerase chain reaction; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>.

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come forth that COX-2 is constitutively expressed in certain tissues (renal epithelia and, particularly, the macula densa, vascular endothelium, central nervous system, ovaries, and uterus).<sup>7-9</sup> Furthermore, Chandrasekharan et al.<sup>10</sup> have reported the presence of a new COX-3 isoform in certain tissues (e.g., cerebral cortex and heart) that is an alternatively spliced variant of the COX-1 gene in which intron 1 is retained.

On the basis of much of the evidence described previously, a hypothesis was originally put forth that NSAIDs primarily induce GI injury by inhibiting the housekeeping COX-1 isoform present in the upper GI tract and that this form of drug-induced ulceration and bleeding could be prevented by the development of drugs that selectively inhibit COX-2.<sup>3,11-13</sup> This concept was readily accepted by both academia and the pharmaceutical industry and was the impetus in the development of the blockbuster drugs celecoxib (Celebrex; Pfizer, New York, NY) and rofecoxib (Vioxx; Merck, West Point, PA). The success of the selective COX-2 inhibitors was, in part, supported by evidence that these drugs induced fewer GI endoscopically observable erosions and ulcers in both volunteers and arthritic patients than did nonspecific COX inhibitors (diclofenac, ibuprofen, and naproxen).<sup>14,15</sup> The evidence from much larger long-term (1-year) outcome studies on patients with arthritis has been less convincing and more controversial; celecoxib did not show a significant difference in GI bleeding, obstruction, or perforation in comparison with ibuprofen and diclofenac in the Celecoxib Long-term Arthritis Safety Study (CLASS) study,<sup>16</sup> whereas rofecoxib showed improved GI safety in comparison to naproxen in the Vioxx Gastrointestinal Outcomes Research study.<sup>13,17</sup> One of the major differences in the 2 studies was that the CLASS study did not exclude low-dose aspirin users, whereas the Vioxx Gastrointestinal Outcomes Research study did. It is interesting to note that the latter study showed, quite unexpectedly, a higher incidence (3-5-fold) of cardiac events (thrombosis, myocardial infarction, angina, and stroke) in elderly subjects who chronically took rofecoxib in comparison to the naproxen comparator over the 1-year study period.<sup>17,18</sup>

The concept that NSAIDs induce GI injury by targeting COX-1, whereas COX-2 plays no role in mucosal defense, has also been challenged by other studies, which have shown that the selective COX-1 inhibitor SC-560 induces little or no gastric injury in laboratory animals.<sup>19-21</sup> Also, studies by Langenbach et al.<sup>22,23</sup> showed that COX-1 knockout mice were not any more suscep-

tible to GI injury than their wild-type littermates. This evidence, originally presented by Wallace et al.<sup>19</sup> and confirmed by others,<sup>20,21</sup> also seems contrary to the original hypothesis. In these studies, it was shown that although neither selective COX-1 or COX-2 inhibitors induce GI injury on their own in laboratory animals, the administration of these drugs in combination results in marked GI injury comparable to that seen with nonselective COX inhibitor indomethacin. These observations therefore may provide an explanation for why patients in the CLASS study, who were allowed to take low-dose aspirin along with celecoxib, did not have evidence of improved GI safety with the selective COX-2 inhibitor.<sup>16</sup>

Finally, there is a growing body of evidence that NSAIDs can induce GI injury by mechanisms independent of COX inhibition.<sup>24,25</sup> There is strong evidence that a number of NSAIDs, or derivatives thereof, with weak or negligible COX-inhibitory activity (e.g., salicylic acid) have the capacity to induce GI injury in laboratory animals and humans. Also, numerous cases can be cited wherein NSAID-induced COX inhibition and GI injury can be separated with regard to dose or time dependence or contrasting routes of administration.<sup>25-29</sup> For example, Ligumsky et al.<sup>30</sup> showed that although intragastrically and parenterally administered aspirin inhibited gastric prostaglandin biosynthesis in a comparable fashion (>95%), only the former induced significant gastric injury to the stomachs of rats. Support for the concept that NSAIDs induce topical injury to the gut by a mechanism independent from COX-inhibition was discussed in more detail in a recent editorial, together with evidence that one of the mechanisms by which these drugs may act is by attenuating the hydrophobic acid-resistant surface properties of the upper GI tract.<sup>25</sup> To explore this concept in more detail, we designed this study to investigate the mechanism of gastric injury by acidified aspirin in COX-1 and COX-2 knockout mice. In addition to studying the effects of the aspirin on mucosal integrity in these transgenic animals, we also investigated the effects of the NSAID on prostaglandin metabolism and surface hydrophobicity of the gastric mucosa and assessed the gastroprotective activity of aspirin preassociated with the phospholipid phosphatidylcholine (PC).

## Materials and Methods

### Animal Breeding and Genotyping

Breeding pairs of mice with targeted disruption of genes encoding COX-1 or COX-2 (*Ptgs-1* and *Ptgs-2*) were

maintained in an outbred mouse colony with a 129/Ola C57BL/6 genetic background, as originally described by Langenbach et al.<sup>22,23</sup> and Morham et al.<sup>31</sup> The mice were housed and bred in the Animal Care Center of The University of Texas Health Sciences Center, where they were on a 12:12-hour light dark cycle and received standard mouse food and water ad libitum. Heterozygous pairs of mice carrying a mutation in either the COX-1 (+/-) or COX-2 (+/-) gene were bred, and their progeny were genotyped. For genotyping by polymerase chain reaction (PCR) analysis, the DNA was isolated from 5-mm sections that were cut from the tails. The tissue was digested overnight at 55°C in a 50 mmol/L Tris (pH 7.5), 100 mmol/L ethylenediaminetetraacetic acid, 100 mmol/L NaCl, and 1% sodium dodecyl sulfate lysis buffer with the addition of proteinase K at 0.5 mg/mL. After digestion, saturated NaCl was added, and the mixture was centrifuged (10,000g); the supernatant was then removed from the pelleted lipid and flocculent protein. The genomic DNA was precipitated by the addition of absolute ethanol. The DNA was collected and re-dispersed in Tris buffer. Sodium acetate (3 mol/L) was added, and the DNA was reprecipitated by 70% ethanol. The DNA pellet was dissolved for PCR analysis in Tris buffer. The isolated genomic DNA was then characterized by PCR to confirm the animal as homozygous for the knocked-out COX-1 or COX-2 gene. The genomic DNA was characterized for the COX-1 genotype by the COX-1 knockout forward primer sequence 5'-GCA GCC TCT GTT CCA CAT ACA C-', the COX-1 wild-type primer 5'-AGG AGA TGG CTG CTG AGT TGG-3', and the reverse primer 5'-AAT CTG ACT TTC TGA GTT GCC-'. DNA was characterized for COX-2 genotype by the COX-2 knockout forward primer 5'-ACG CGT CAC CTT AAT ATG CG-', the COX-2 wild-type forward primer 5'-ACA CAC TCT ATC ACT GGC ACC-', and the COX-2 reverse primer 5'-ATC CCT TCA CTA AAT GCC CTC-'. The thermocycler was programmed for 1 cycle at 94°C for 1 minute and 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds and was then held at 4°C. The COX-1 wild-type band was 601 base pairs (bp). The COX-1 knockout band was 646 bp. The COX-2 wild-type band was 760 bp. The COX-2 knockout band was 905 bp.

### Prostaglandin Radioimmunoassay

Flash-frozen, full-thickness samples of the oxyntic glandular region of the stomach from control and test animals were weighed, and the prostaglandin was extracted by methanol. The samples were homogenized in methanol. The tissue was removed by centrifugation. The dissolved prostaglandin was isolated by blowing off the methanol under nitrogen gas. The prostaglandin was re-dissolved in 1 mL of 50 mmol/L Tris buffer, pH 7.4. The prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) levels were assessed by radioimmunoassay with αPGE<sub>2</sub> antibody from Sigma Scientific (P-5164; St. Louis, MO) and radioactive antigen tracer from Amersham (TRK 431; Piscataway, NJ), as

previously described.<sup>32</sup> Prostaglandin measurements were corrected for weight differences.

### Analysis of COX Messenger RNA by Reverse-Transcription PCR

Quantitative real-time reverse-transcription PCR was performed with a 7700 Sequence Detector (Applied Biosystems, Foster City, CA).<sup>33,34</sup> Specific quantitative assays for COX-1 and COX-2 were developed by using Primer Express software (Applied Biosystems) by following the recommended guidelines based on sequences from GenBank. Total RNA was extracted from gastric mucosa by homogenization in RNAsol B (Tel-test, Houston, TX) and extraction according to the manufacturer's instructions. The resulting aqueous phase was then further purified on an RNeasy spin column (Qiagen, Valencia, CA). Complementary DNA was synthesized by combining 400 nmol/L assay-specific reverse primer, 500 μmol/L deoxynucleotides, Superscript II buffer, dithiothreitol, and 10 U of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) in a 96-well plate followed by sample (25 ng/μL). Plates were incubated in a thermocycler (MJR, Waltham, MA) for 30 minutes at 50°C followed by 72°C for 10 minutes. Subsequently, a PCR master mix (400 nmol/L forward and reverse primers, 100 nmol/L fluorogenic probe, 3 mmol/L MgCl<sub>2</sub>, 200 μmol/L deoxynucleotides, PCR buffer, and 1.25 U of *Taq* polymerase [Invitrogen]) was added directly to each well of the complementary DNA plate. The plate was run in the 7700 by using the following cycling conditions: 95°C for 1 minute, 40 cycles at 95°C for 12 seconds, and 60°C for 1 minute. The resulting data were analyzed with SDS software (Applied Biosystems) with tetramethylrhodamine as the reference dye. The amount of RNA added to a reverse-transcription PCR for each sample was determined by measuring the β-actin transcript levels in each sample. The final data were normalized to β-actin and are presented as the molecules of transcript/molecules of β-actin × 100.

### Analysis of COX Protein by Western Blot Analysis

Flash-frozen gastric samples were homogenized in lysis buffer (5% sodium dodecyl sulfate, 0.5% Triton X-100, 2 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktail; Sigma Chemical Co., St. Louis, MO). After homogenization, the samples were boiled for 5 minutes and then centrifuged at 12,000g for 30 minutes. The supernatant was used for Western blot analysis. Protein content was determined with the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Equal amounts of protein (100 μg) were electrophoresed onto NuPAGE Bis-Tris Gel 4%–12% (Invitrogen) and electroblotted onto polyvinylidene difluoride membranes. After washing in deionized water, the membranes were blocked for at least 1 hour in 5% nonfat dry milk at room temperature and probed overnight at 4°C with the antibody for COX-2 (Cayman Chemical, Ann Arbor, MI) at a 1:1000 dilution. The membranes were incubated with secondary antibody (anti-rabbit

immunoglobulin G conjugated with horseradish peroxidase from Amersham; 1:2000 dilution). Detection was made with an enhanced chemiluminescence reagent (Amersham), and the membranes were exposed to x-ray film for visualization.

### Gastric Lesion Score

Mice were fasted overnight, and the next morning they were challenged with 20 mmol/L of aspirin (pH 3.5) dissolved in 0.2 mL of saline or an equivalent volume of vehicle alone. In 1 experimental series, the required amount of aspirin was weighed and combined with an equal weight of triple-strength lecithin oil (Phosal 35 SB; American Lecithin, Oxford, CT) containing 35% PC and mixed under mild heat (40°C) before intragastric administration as an aqueous suspension. Fifteen minutes later, the mice were intragastrically administered either 0.2 mL of saline or 0.7N HCl, and after 1 hour the animals were killed by CO<sub>2</sub> inhalation. The stomachs were removed, cut open along the greater curvature, and rinsed in saline, and the length and width of the hemorrhagic gastric lesions were scored by caliper with a previously described technique by an observer blinded to the experimental conditions.<sup>35</sup>

### Measurement of Mucosal Surface Hydrophobicity

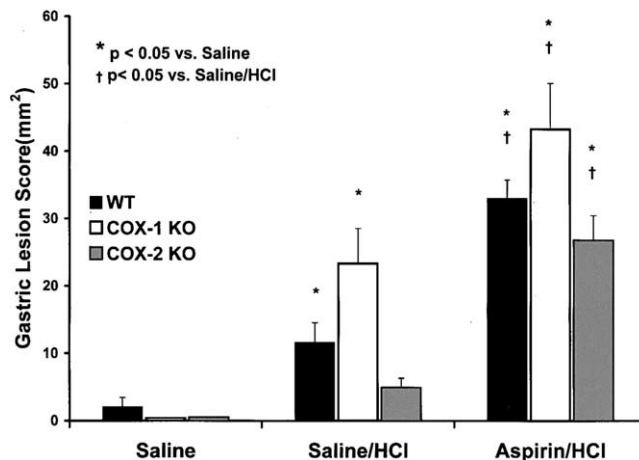
Gastric mucosal tissue was dissected from the oxyntic mucosa, rinsed in cold saline, and placed mucosal side up on the stage of a goniometer (Rame-Hart, Mountain Lakes, NJ). Residual water was removed from the surface of the mucosa by blotting with filter paper, and tissue was air-dried for 30 minutes. The surface hydrophobicity of the mucosa was then measured by the application of a 10- $\mu$ L droplet of water on the mucosa with the use of micromanipulators, and the contact angle that formed at the air/liquid/solid interface was measured under a telescopic eyepiece. The technique used to measure the contact angle on GI mucosa has been described in detail in previous articles from our laboratory and others, as has an assessment that the mucosal structure remains preserved at the time the measurement is made.<sup>35–37</sup>

### Light Microscopy

At death, a biopsy sample of gastric tissue was placed in buffered formalin, embedded in formalin, cut into 4- $\mu$ m sections, and stained with H&E under light microscopy.

### Statistical Analysis

Intergroup comparisons were performed by analysis of variance, followed by Fisher's least significant difference test for significance, with  $P < 0.05$  as the limit for significance. Linear regression analyses were also performed to determine whether a significant association was present between the gastric lesion score, and gastric mucosal PGE<sub>2</sub> concentration, and surface hydrophobicity.



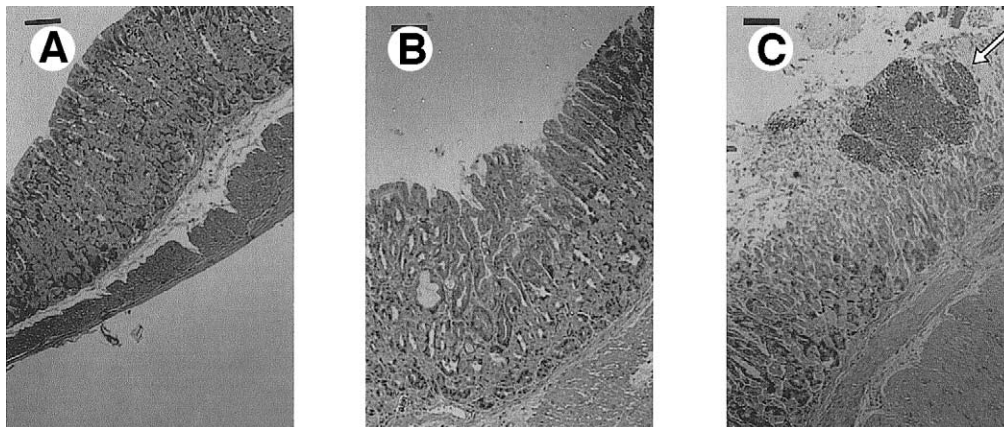
**Figure 1.** Lesion score as a measure of gastric damage in wild-type (WT) and COX knockout (KO) mice. Note that the COX-1 KO mice seemed most sensitive to the damaging actions of HCl alone and aspirin in combination with HCl;  $n = 4\text{--}5$  rats per group.

## Results

### Ulcerogenic Effect of Aspirin/HCl in COX Knockout and Wild-Type Mice

In the initial series of experiments, we challenged fasted COX-1-null and COX-2-null mice with 20 mmol/L aspirin followed 15 minutes later with 0.7N HCl. One hour later, the stomachs were examined, and the surface area of the hemorrhagic lesions was scored. In this experiment, we also measured the ulcerogenic activity of 0.7N HCl alone in these animal groupings. Figure 1 shows that in the absence of the NSAID, HCl caused significant gastric injury in the wild-type and COX-1-null mice: the ulcer score was higher in the mice deficient in the COX-1 isoform. In contrast, COX-2-null mice seemed to have greater resistance to luminal acid, because the lesion score was not significantly different from saline-treated values. Furthermore, aspirin in combination with HCl induced marked gastric ulceration in all animal groups that was significantly worse than the injury induced by HCl alone. COX-1-null mice had a tendency to have greater injury, although this intergroup difference was not statistically significant. It also should be noted that the stomachs of saline-treated control rats were essentially devoid of any gastric injury.

Examination of the tissue under light microscopy revealed that 0.7N HCl induced only mild surface injury to the oxyntic mucosa of mice in all groups, whereas the glandular architecture remained intact. In contrast to this pattern, pretreatment with aspirin 15 minutes before exposure to strong acid induced extensive injury to the gastric mucosa which extended to the glandular mucosa and occasionally involved the muscularis mucosa



**Figure 2.** Micrographs depicting the light microscopic structure of oxyntic mucosa of COX-1 knockout mice intragastrically treated with (A) saline, showing intact surface and glandular architecture; (B) 0.7N HCl, showing surface cell injury and exfoliation of mucous cells lining the surface and neck region; and (C) aspirin/HCl, showing a necrotic ulcerogenic lesion extending to basal aspects of the gland with evidence of bleeding, cell exfoliation from all levels of the mucosa, and neutrophil extravasation. Arrow points to an area containing extruded cellular debris adherent to thrombotic plaques (bar = 100 μm).

(Figure 2). Both the acid-induced surface injury to the gastric mucosa and the necrotic ulcerogenic lesions caused by the aspirin/HCl challenge seemed more severe in the COX-1 knockout mice than in the wild-type or COX-2-null animals.

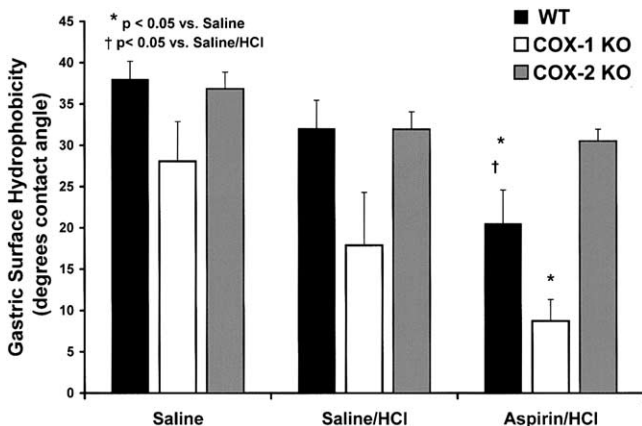
**Effect of Aspirin/HCl on Gastric Mucosal Surface Hydrophobicity in COX Knockout and Wild-Type Mice**

Figure 3 shows the effects of HCl alone and in combination with aspirin on the surface hydrophobicity of the gastric mucosa as measured by contact angle analysis. It can be appreciated that the gastric mucosa of saline-treated wild-type mice had modest hydrophobic

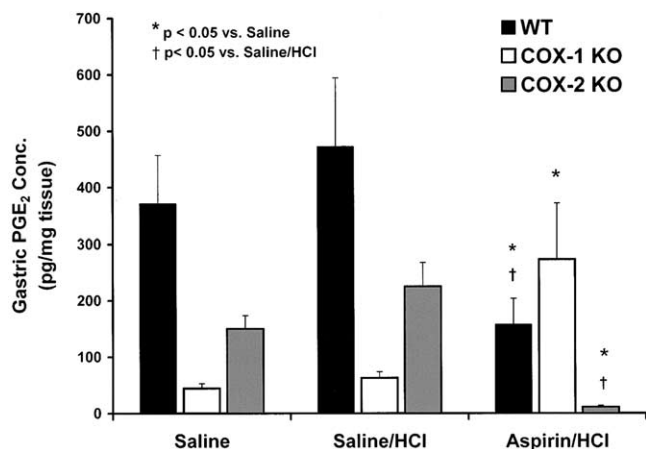
properties, as shown previously,<sup>38</sup> with contact angle readings ranging from 30° to 40°. Although challenge with strong luminal acid had little effect on the surface hydrophobicity of wild-type and COX-2-null mice, there was a tendency for HCl to induce a 40%–50% decrease in contact angles of COX-1 knockout mice in comparison to the saline control values. Furthermore, the decrease in mucosal surface hydrophobicity was more pronounced and reached statistical significance in both COX-1-null and wild-type mice that were challenged with the aspirin/HCl combination, whereas no significant attenuation in gastric contact angle readings was recorded in COX-2-null animals.

**Effect of Aspirin/HCl on Gastric Prostaglandin Concentration in COX Knockout and Wild-Type Mice**

As expected, the concentration of the eicosanoid PGE<sub>2</sub> in the gastric mucosa was low in the COX-1-null mice (~12% of the values in wild-type mice), thus reflecting the prominent role of this COX isoform in the constitutive maintenance of tissue prostaglandin levels (Figure 4). It should be noted that this difference was not as great as the 99% reduction reported in a previous article<sup>22</sup>; this most likely is attributable to differences in the methods of prostaglandin extraction and analysis performed by the 2 laboratories. It is interesting to note that the mucosal levels of PGE<sub>2</sub> were also reduced to less than the wild-type levels in COX-2-deficient mice (~40% of wild-type values). This suggests that this isoform may have significant constitutive activity as well. Although mucosal PGE<sub>2</sub> levels did not change after challenge with strong luminal acid, the combination of



**Figure 3.** Gastric contact angles were measured as an index of mucosal surface hydrophobicity. Both wild-type (WT) and COX-1 knockout (KO) mice had a significant reduction in mucosal surface hydrophobicity after intragastric challenge with aspirin in combination with HCl. In contrast, this gastric surface property was not affected in COX-2 knockout mice; n = 4–15 rats per group.



**Figure 4.** Effect of treatments on gastric PGE<sub>2</sub>, an index of COX activity. Note that aspirin treatment in combination with HCl induced an approximately 60% decrease in gastric prostaglandin levels in wild-type (WT) mice and a >90% inhibition in COX-2-null mice. In contrast, mucosal PGE<sub>2</sub> was not inhibited by aspirin/HCl treatment and showed a tendency to increase in COX-1 knockout (KO) mice; n = 6–15 rats per group.

aspirin with HCl induced a significant decrease in mucosal prostaglandin concentration in both wild-type and COX-2-null mice; the levels of the latter group decreased to undetectable values. In sharp contrast with this pattern, the mucosal prostaglandin levels actually increased above saline-treated values in COX-1-null mice that were exposed to the aspirin/HCl combination.

#### Effect of Aspirin/HCl on Messenger RNA COX Expression in Wild-Type and COX Knockout Mice

By use of real-time PCR, the expression of COX-1 and COX-2 messenger RNA (mRNA) was assessed under the previously described experimental conditions. These results are graphically depicted in Figure 5. It can be appreciated that the expression of COX-1 mRNA seemed to be unaffected by HCl alone and in combination with aspirin in wild-type and COX-2-null mice (Figure 5B). In contrast, the expression of mRNA for COX-2 seemed to be modestly, but not significantly, increased by exposure to luminal acid. Furthermore, the combination of HCl and aspirin induced a significant increase in COX-2 mRNA in both wild-type and COX-1-null mice over their respective saline-treated control values (Figure 5A). The latter group showed a marked 3–4-fold increase in message expression.

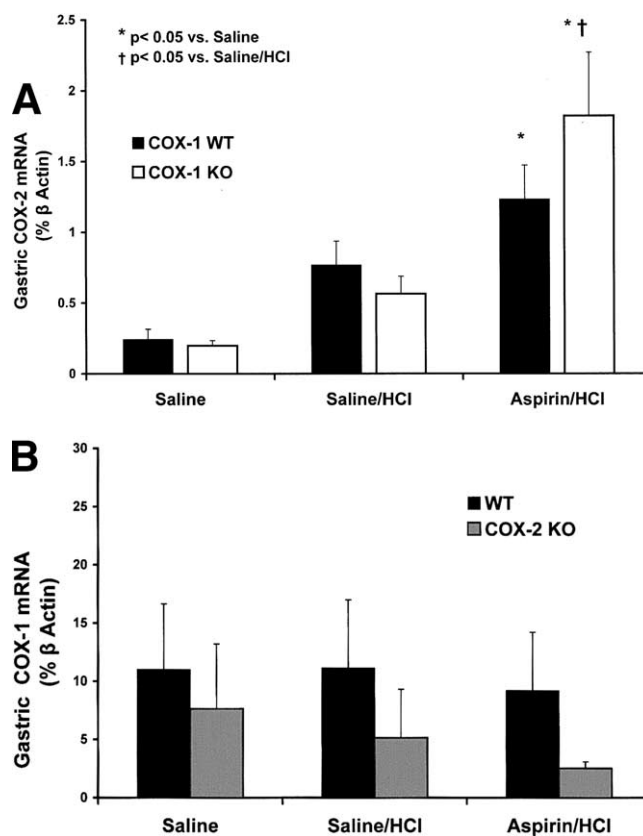
#### Effect of Aspirin/HCl on Gastric COX-2 Protein by Western Blot Analysis

The levels of COX-2 in gastric tissue were determined by Western blot analysis of samples obtained 1

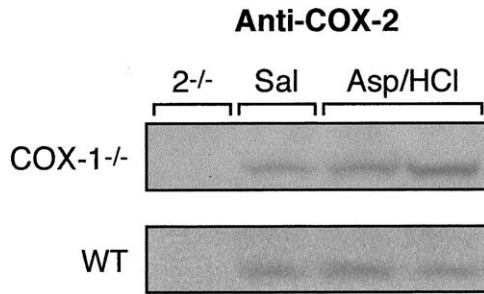
hour after gavage with saline or aspirin/HCl. In the COX-1 knockout mice, COX-2 protein was increased 50%–100% in the aspirin/HCl-treated animals compared with the saline-treated mice (Figure 6, upper panel). However, in the samples from the wild-type mice, comparable changes in the levels of COX-2 protein at 1 hour after treatment were not observed (Figure 6, lower panel).

#### Gastroprotective Role of Phosphatidylcholine/Aspirin in COX-1 Knockout Mice

Table 1 describes results in which COX-1 knockout mice were challenged with HCl, aspirin/HCl, or PC/aspirin/HCl. It can be appreciated that the aspirin-induced increase in the gastric lesion score was prevented if the animals were challenged with PC/aspirin in conjunction with HCl.



**Figure 5.** COX mRNA levels in the gastric tissue (expressed as percentage of  $\beta$ -actin mRNA) were determined by quantitative reverse-transcription PCR. (A) Note that COX-2 mRNA levels in wild-type (WT) and COX-1 mice, which showed a tendency to increase with HCl alone, were significantly increased after intragastric challenge with aspirin in combination with HCl. (B) In contrast, gastric COX-1 mRNA levels were unchanged in response to intragastric challenge with HCl alone and in combination with aspirin; n = 4–11 rats per group. KO, knockout.



**Figure 6.** Western blot analysis showing the effects of aspirin (Asp) in combination with HCl treatment on COX-2 protein levels in gastric samples obtained 1 hour after treatment. The *upper panel* shows a 50%–100% increase of COX-2 protein in the COX-1 knockout mice, in contrast to the modest to negligible changes of COX-2 protein observed in wild-type (WT) samples (*lower panel*). Gastric samples from COX-2 knockout mice (2<sup>-/-</sup>) are shown as negative controls. Sal, saline.

## Discussion

The results presented previously, with transgenic mice deficient in either COX-1 or COX-2, confirm earlier studies showing that the absence of 1 isoform of COX does not predispose an animal to spontaneous gastric ulceration.<sup>22,23</sup> However, a tendency was observed for COX-1-null mice to be more sensitive to the ulcerogenic actions of strong luminal acid alone or in combination with aspirin, an irreversible nonselective COX inhibitor. These results differed from those of the earlier study by Langenbach et al.,<sup>22</sup> who reported that indomethacin-induced gastric injury was less frequent in COX-1-null mice. The reason for these contrasting findings is uncertain but may relate to the fact that the drug used in this study (aspirin) has a more topical mode of action than most conventional NSAIDs, including indomethacin. It is interesting to note that COX-2 knockout animals, which selectively express only COX-1, seemed to be resistant to the ulcerogenic actions of strong acid, although they did develop gastric lesions when pretreated with aspirin before acid challenge.

It is also important to point out that because rats and mice are very resistant to the ulcerogenic actions of aspirin, it has been our and other investigators' experience that to induce a macroscopically observable and quantifiable lesion, one has to either administer aspirin at very high dosages (100–250 mg/kg) in physiological concentrations of acid (0.15N) or intragastrically deliver the NSAID at a lower dose (18 mg/kg) in conjunction with a high concentration of HCl (0.6–0.75N). We used this technique in this study and previously published studies.<sup>35</sup> We believe that the latter approach has a number of advantages in that the lower dose of aspirin more closely simulates its concentration (20 mmol/L) in the gastric juice of humans after the ingestion of 2

aspirin tablets, and it allows one to obtain valuable data on the gastric barrier to luminal acid both under control conditions and when the barrier is attenuated by a damaging agent. It also should be noted, as shown in Figure 2, the administration of aspirin at the lower dose followed by challenge with strong acid induces a necrotic ulcerogenic lesion that affects the distal glandular mucosa and may extend to the submucosal layer. Furthermore, this ulcer injury is dependent on aspirin pretreatment; light microscopy revealed that the damage induced by 0.7N HCl alone was restricted to the apical surface of the oxyntic mucosa, except in COX-1 knockout mice, in which deeper injury was occasionally observed.

A number of conclusions can be drawn from these results. First, it seems clear that COX-1 is not required for the normal maintenance of the gastric barrier, although it does seem to play an important permissive protective role against luminal acid. Second, the fact that aspirin induced significant gastric injury in COX-1-null mice runs contrary to the original concept that the inhibition of COX-1 was the primary cause of gastric injury induced by NSAIDs and that COX-2 played little or no role in gastric defense.<sup>2,3,6,11–13</sup> Indeed, the original concept has been challenged by other studies on COX knockout animals and by recent studies showing that selective COX-1 inhibitors induced little or no gastric damage in laboratory animals, as did selective COX-2 inhibitors, whereas using both selective inhibitors in combination induced injury comparable to that seen with a nonspecific COX inhibitor.<sup>19–21</sup>

A third conclusion is that the COX-2 isoform is constitutively expressed in the gastric mucosa and that this expression can be induced by damaging agents such as luminal acid and aspirin, with marked increases in COX-2 mRNA and protein expression when the 2 injurious agents are given in combination. It should be noted that under our experimental conditions, the aspirin-induced increase in COX-2 mRNA was more prominent than the increases in COX-2 protein, as determined by Western blot analysis. This apparent discordance may be attributable to the fact that the

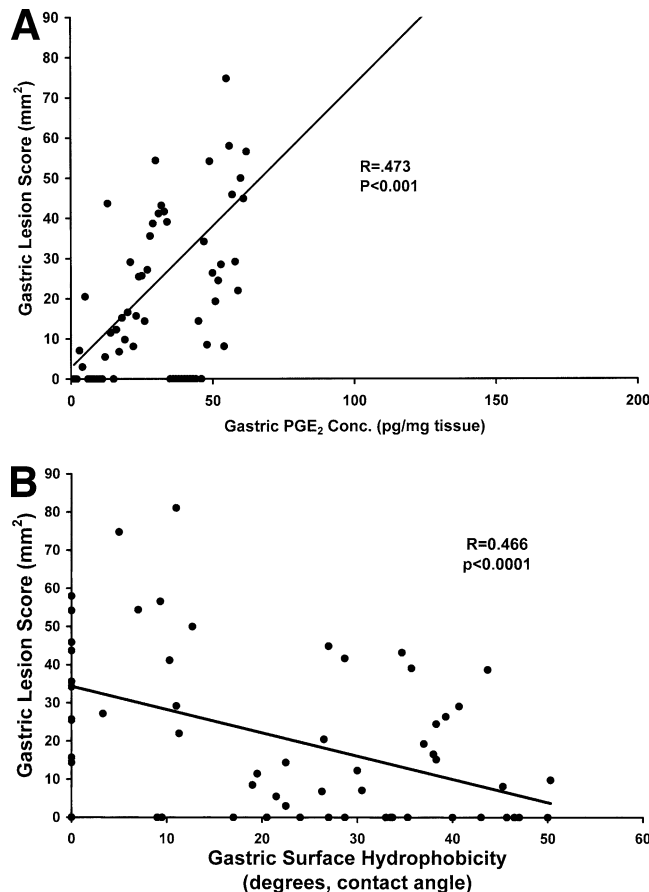
**Table 1.** Protective Effect of PC/Aspirin Versus Aspirin in COX-1-Null Mice

Group	Gastric lesion score (mm <sup>2</sup> )
Saline/HCl	18.1 ± 4.1
Aspirin/HCl	36.9 ± 5.7 <sup>a</sup>
PC/aspirin/HCl	10.0 ± 1.6 <sup>b</sup>

<sup>a</sup>*P* < 0.05 vs. saline/HCl group; <sup>b</sup>*P* < 0.05 vs. aspirin/HCl group; n = 5 rats per group.

gastric tissue was collected at an early time point (1 hour) after NSAID challenge, thus limiting the period for translation of the COX-2 protein. This is not the first indication that COX-2 expression in the GI mucosa is induced by cytokines, ulcerogenic agents (including NSAIDs), and COX-1-selective inhibitors in particular.<sup>21,39,40</sup> The cellular origin of increases in COX-2 mRNA and protein has yet to be clearly defined, but the most compelling data indicate a prominent role of infiltrating lymphocytes and fibroblastic cells of the lamina propria.<sup>40–43</sup> Indeed, a recent report by Mifflin et al.<sup>44</sup> indicates that aspirin promotes COX-2 expression of intestinal myofibroblasts in the presence of interleukin-1, primarily by stabilization of COX-2 mRNA, by a yet-to-be-defined process mediated by the activation of p38, a stress-activated mitogen-activated protein kinase. This interesting observation was based on earlier studies of Schwenger et al.<sup>45</sup> that salicylates activate p38 and on a recent study that a selective inhibitor of p38 significantly reduces the half-life of the COX-2 message of interleukin-1-activated intestinal myofibroblasts from 1 hour to <20 minutes.<sup>43</sup>

Our evidence that the concentration of PGE<sub>2</sub> in the gastric mucosa actually increases in COX-1-null mice that are challenged with aspirin/HCl suggests that the increase in COX-2 mRNA may be sufficient to produce enough COX-2 enzyme, by mass action, to partially overcome the irreversible inhibitory effects of aspirin. We speculate further that such an escape mechanism of COX-2 from the inhibitory actions of aspirin also occurs in wild-type animals and may be why aspirin induces only a modest ~60% decrease in mucosal prostaglandin concentration, as shown in Figure 4. It can be argued that the increase in COX-2 expression may be a compensatory response to increase the levels of gastroprotective prostanoids during periods of gastric injury. If this is the case, then one has to be concerned that the administration of selective COX-2 inhibitors to animals or humans may be contraindicated under certain conditions in which there is pre-existent gastric injury and mucosal repair and angiogenesis need to occur. In support of this possibility, a number of studies have reported that selective COX-2 inhibitors exacerbate ulcer healing and ischemia/reperfusion injury to the gut and attenuate the tissue's ability to respond to mild damaging agents.<sup>39–41,46,47</sup> Another explanation comes from recent studies by Fiorucci et al.<sup>48</sup> that COX-2 catalyzes the generation of 15(R)-epilipoxin A<sub>4</sub> in the presence of aspirin, which inhibits neutrophil recruitment to the site of inflammation, and this protective mechanism is blocked by COX-2-selective inhibitors.



**Figure 7.** Regression analysis evaluating the association between gastric lesion formation and (A) gastric PGE<sub>2</sub> and (B) mucosal surface hydrophobicity. Note that the gastric injury score seemed to paradoxically increase in association with gastric prostaglandin levels, whereas it was significantly associated with a decrease in mucosal hydrophobicity; n = 62 rats. Conc, concentration.

An alternative possibility that needs to be considered is that multiple protective mechanisms are operative in the maintenance of the gastric mucosal barrier and that NSAIDs may induce gastric injury, in part, by a mechanism independent from COX inhibition and a depletion of tissue prostaglandin levels. Indeed, the paradoxical correlation between mucosal PGE<sub>2</sub> concentration and gastric injury in this study (in which both properties seemed to increase in an associated fashion), depicted in Figure 7A supports the role of COX in the gastric mucosa's ability to recover from injury by NSAIDs, as opposed to playing a role in the pathogenic mechanism of these drugs—where a negative correlation would be expected. A particularly obvious example of the lack of a causal linkage between these 2 properties comes from comparing the effects of aspirin/HCl in COX-1 and COX-2 knockout mice. Inspection of Figures 1 and 4 shows that COX-1-null mice had significantly greater injury than observed in COX-2-null mice in response to

challenge with aspirin/HCl ( $43 \pm 7 \text{ mm}^2$  vs.  $27 \pm 4 \text{ mm}^2$ ), whereas the gastric concentration of PGE<sub>2</sub> was 20 times higher in COX-1-null mice than in their COX-2-null littermates.

One possibility is that NSAIDs may cause topical injury to the GI mucosa by a mechanism not directly linked to COX inhibition. This subject has been discussed in detail in several recent review articles.<sup>24,25</sup> One mechanism that our laboratory has explored is the ability of NSAIDs to attenuate the hydrophobic barrier properties of the mucosal surface, making it more susceptible to attack by luminal acid. In these studies, we used contact angle analysis to show that aspirin and other NSAIDs have the capacity to induce a marked decrease in surface hydrophobicity in rodent and canine gastric mucosa in a time- and dose-dependent fashion both *in vivo* and *in vitro*.<sup>35-37</sup> It is interesting to note that regression analysis of lesion score vs. gastric contact angles, including all animals in the study (wild-type and COX-null mice), showed a significant association between these 2 properties. The most marked decrease in surface hydrophobicity occurred in COX-1-null mice, which also experienced the greatest gastric injury in response to the aspirin/HCl challenge (Figure 7B). It is, however, important to note that this apparent linkage was not observed in COX-2-null mice that maintained normal gastric surface hydrophobicity under all experimental conditions, including aspirin/HCl treatment, in which significant gastric injury was sustained.

We have also obtained evidence that NSAIDs may induce this transition of the gastric mucosa to a wettable state by chemically associating with and destabilizing an extracellular lining of surface phospholipids that coat the mucous gel layer and provide the tissue with its surface hydrophobic properties.<sup>35-37</sup> One way to prevent this molecular interaction from happening is to preassociate a PC molecule with the NSAID before administration. Table 1 shows that, in support of previous studies performed in our laboratory on nontransgenic animals,<sup>35</sup> PC/aspirin in combination with HCl induced little or no gastric injury in COX-1 knockout mice, in comparison to the injurious effect of unmodified aspirin.

It should also be noted that we have previously shown that exogenous prostaglandin administration can increase gastric mucosal surface hydrophobicity and phospholipid metabolism of rodent and canine gastric mucosa.<sup>49-51</sup> In this study, however, it was found that mucosal contact angle readings could be maintained at control values in the face of a low-negligible mucosal prostaglandin concentration. This conclusion was supported by the finding that mucosal hydrophobicity was not changed in COX-2

null mice challenged with aspirin/HCl from the values of saline-treated animals, although the gastric mucosal PGE<sub>2</sub> concentration was decreased by >90% below saline-treated values. This indicates that mucosal surface hydrophobicity can be controlled and maintained by mechanisms independent from the prostaglandin/COX pathway.

In conclusion, we have shown that aspirin can induce gastric injury in transgenic mice deficient in either COX-1 or COX-2, suggesting that a simple inhibition of COX-1 activity cannot provide the only explanation for NSAID-induced gastric pathogenesis. It is interesting to note that challenge with aspirin/HCl resulted in a significant increase in COX-2 mRNA, protein, and PGE<sub>2</sub> concentrations in COX-1-null mice. This provides evidence for a role of the COX-2 isoform in the maintenance of mucosal prostanoid levels and repair, even in the presence of a COX-1-selective NSAID, such as aspirin. We also presented evidence that the linkage between gastric ulceration induced by aspirin/HCl and gastric prostaglandin concentration was more consistent with the possibility that COX plays a role in the mechanism of recovery from NSAID injury, rather than in its pathogenesis. In contrast, the negative association between NSAID-induced gastric injury and mucosal surface hydrophobicity is consistent with the possibility that NSAIDs topically injure the mucosa by increasing the tissue's wettability to luminal acid. The role of surface phospholipids is the mechanism by which aspirin disrupts the gastric mucosal barrier was further supported by evidence that aspirin-induced gastric injury in COX-1 knockout mice could be prevented if the animals were administered PC/aspirin.

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