Role of the different isoforms of cyclooxygenase and nitric oxide synthase during gastric ulcer healing in cyclooxygenase-1 and -2 knockout mice

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Cyclooxygenase (COX), exists in at least two isoforms (39). COX-1 is constitutively expressed in the gastrointestinal tract and many other tissues, whereas COX-2 is predominantly expressed in inflammatory and neoplastic tissues (39, 42). This finding led to the development of selective COX-2 inhibitors aimed at reducing PG-dependent inflammation while leaving protective gastric PG synthesis intact (39). Compared with traditional NSAIDs, COX-2-selective inhibitors showed significantly decreased formation of gastric ulcers and reduced gastrointestinal side effects (28). However, this advantage was lost if aspirin was coadministered with selective COX-2 inhibitors (28). In addition, COX-2-selective inhibitors delayed the healing of human gastric ulcers (6). Furthermore, increased cardiovascular events were observed in patients treated with selective COX-2 inhibitors (3).

These clinical studies confirmed earlier animal studies, suggesting that selective COX-2 inhibitors retain several relevant side effects of the traditional NSAIDs (19, 22, 23). In rodent gastric ulcer healing, both traditional NSAIDs and selective COX-2 inhibitors decreased epithelial cell proliferation and angiogenesis and delayed ulcer healing (19, 23, 25). However, these studies were unable to differentiate between COX-independent and COX-2-specific side effects of the selective COX-2 inhibitors (38). Furthermore, it is unclear whether COX-2-selective inhibitors delay ulcer healing less than traditional NSAIDs (23). Moreover, no reliable data are available on the role of COX-1 during gastric ulcer healing. The development of selective COX-1 and COX-2 inhibitors as well as COX-1 and COX-2 knockout mice has now allowed the functional role of COX-1 and COX-2 to be studied (1, 8, 15, 20, 27, 29).

Nitric oxide (NO) is produced by NO synthase (NOS) from l-arginine. NOS inhibitors delay, and NO donors enhance healing (14), suggesting relevant roles for NOS in gastric ulcer healing. There are three isoforms of NOS, namely neuronal NOS (NOS-1), inducible NOS (NOS-2), and endothelial NOS (NOS-3). Whereas NOS-1 and NOS-3 are constitutively expressed in the intact tissue, NOS-2 is predominantly expressed at inflammation sites. Although NOS-3 has been shown to play a beneficial role in gastric ulcer healing (17), the role of NOS-2 in healing is less clear (17, 37). Relevant interactions between the NOS and COX system have been detected (40), which led to the development of NO-releasing NSAIDs aimed at reduc-
ing NSAID-induced gastrointestinal side effects. In rodents, NO-releasing NSAIDs have been reported as interfering less with gastric ulcer healing than traditional NSAIDs (9); and in humans, COX-inhibiting NO-donators have been reported to have less gastrointestinal toxicity than traditional NSAIDs (12).

We aimed to investigate gastric ulcer healing parameters in COX-1 and COX-2 deficiency and inhibition models and to quantify regulation of the isoforms of COX and NOS mRNA in these healing models. This study further intends to characterize the role of COX-2 by using COX-2 knockout mice and three selective COX-2 inhibitors to assess the role of COX-1 during gastric ulcer healing in the presence and absence of COX-2-derived PGs.

MATERIALS AND METHODS

Animals and materials. The University of Berne’s Committee for Animal Studies approved this study. COX-1−/− and COX-2−/− mice have been described previously (15, 20). Female wild-type COX-1−/− and COX-2−/− mice weighing 20–25 g were purchased from Taconic (Germantown, NY) and studied 2–4 wk after delivery at the age of 9–13 wk. Where a comparison between wild-type and COX knockout mice was carried out, animals were matched for age, weight, and genetic background. Mice were kept under normal laboratory conditions with free access to water and a standard chow. SC-560 [a selective COX-1 inhibitor (29)], celecoxib, rofecoxib, and valdecoxib (3 selective COX-2 inhibitors), and piroxicam (an unselective COX inhibitor) were used in this study. SC-560 was kindly provided by Merck Frosst (Quebec, Canada). Celecoxib, rofecoxib, valdecoxib, and piroxicam were purchased at the pharmacy. The drugs were dissolved in DMSO (final concentration: <10%), which was also used as the vehicle. TRIZol was from Invitrogen (Karlsruhe, Germany), and the primers were from biomers.net (Ulmm, Germany). [3H]Arginine was from Amersham (Buckinghamshire, UK), and the Dowex AG 50WX-8 resins were from Bio-Rad Laboratories ( Hercules, CA). Bromodeoxyuridine (BrdU), MAb against BrdU, and antibodies against von Willebrand factor came from Dako Cytomation (Zug, Switzerland). All other materials were from Sigma.

Induction of gastric cryoulcer. Gastric ulcers were produced with a cryoprobe as previously described (26). In brief, fed animals were anesthetized (0.5 mg/kg medetomidine + 5 mg/kg cloramidol + 0.05 mg/kg fentanyl intraperitoneal), and the abdomen was opened by median incision. The stomach was carefully filled with food from the rumen to standardize gastric wall tension and to prevent freezing of the opposite gastric wall. A cylindrical steel probe (with a flat tip of 2-mm diameter) was held with an isolated clamp, cooled in liquid nitrogen for 15 s, and then placed with soft pressure for 15 s on the posterior wall of the midcorpus gastric wall, causing an impression of the wall of 1 mm. After the lesion was spontaneously thawed, the serosa was rinsed with sterile isotonic saline solution and the abdomen was closed with silk sutures. This method allows reproducible induction of a round ulcer with an area of ~3.9 mm² (as measured 1 day after ulcer induction). The animals recovered well within 30 min of surgery.

Selection of doses of COX inhibitors. The dosing of the drugs was selected on the basis of previous studies (5, 10, 23, 29, 33, 41, 43) and preliminary experiments. Because SC-560 has a shorter half-life than the other COX inhibitors, it was dosed twice daily intragastrically (35). In preliminary studies, COX inhibition, selectivity, and the safety of COX inhibitors were tested in wild-type, COX-1−/−, and COX-2−/− mice. Gastric 6-keto-PGF₁α synthesis in the intact tissue is largely mediated by COX-1 and was used to determine COX-1 inhibition. Ulcer healing in COX-1−/− mice is independent of COX-1 and was used to test the specificity of SC-560 for COX-1 at different doses. At a dose of 2 × 5 mg/kg SC-560, gastric 6-keto-PGF₁α synthesis was inhibited by 89%; at this dose, no COX-1-unrelated effects on gastric ulcer healing were observed in COX-1−/− mice. At a dose of 2 × 37.5 mg/kg SC-560, however, ulcer healing in COX-1−/− mice was inhibited by COX-1-unrelated mechanisms. On the basis of previous studies (29), SC-560 does not inhibit COX-2-mediated inflammatory PG synthesis at a dose of 2 × 5 mg/kg. From these combined data, we selected a dose of 2 × 5 mg/kg SC-560 as the standard dose for most experiments, but we also tested one lower (2 × 1.25 mg/kg) and two higher (2 × 12.5 and 2 × 37.5 mg/kg) doses of SC-560 for ulcer healing studies in wild-type and COX-1−/− mice (see Table 1).

The COX-2-selective drugs celecoxib (1 × 25 mg/kg), rofecoxib (1 × 5 mg/kg), and valdecoxib (1 × 5 mg/kg), and the unselective COX inhibitor piroxicam (1 × 5 mg/kg) have been shown to inhibit COX-2-mediated parameters by >80% in inflammatory (COX-2 mediated) in vivo models (5, 33, 43). In our preliminary studies, intragastric administration of COX-2 selective inhibitors had no effects on gastric 6-keto-PGF₁α synthesis and no significant effects on ulcer healing in COX-2−/− mice. Piroxicam (1 × 5 mg/kg ig) significantly inhibited COX-1-mediated gastric PG synthesis. Furthermore, the selected doses of COX inhibitors were tested for safety and showed no effects on the animal’s well-being.

Selection of time points after ulcer induction. The time points were determined from previous studies in rats (11, 23–26) and preliminary studies using this mouse ulcer model. In preliminary studies in wild-type mice studied on days 1–7, and 10, the mRNA levels of COX and NOS isoforms and epithelial cell proliferation in the mucosal ulcer margin peaked on day 4 and the microvessel density in the ulcer bed peaked on day 7. mRNA levels and epithelial cell proliferation in the ulcer margin showed the most significant differences between wild-type and COX-2−/− mice on day 4. Histological ulcer size, length of the mucosal ulcer margin, thickness of the ulcer bed, and microvessel density in the ulcer bed showed the most significant differences between the groups on day 7. On the basis of these preliminary results, mRNA levels and epithelial cell proliferation were measured on day 4 and microvessel density, length of ulcer margin, and thickness of the ulcer bed were measured on day 7.

Treatment groups, ulcer area and histomorphometric parameters. Mice with cryoulcera were randomly assigned to 12 groups and treated intragastrically for 1–10 days as shown in Table 1. The animals underwent CO₂ anesthesia, and laparotomy was performed.

Table 1. Treatment groups

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>Genotype (mice)</th>
<th>Therapy</th>
<th>Dose, mg/kg·day⁻¹·ig</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>68</td>
<td>Wild-type</td>
<td>Vehicle</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>COX-1−/−</td>
<td>Vehicle</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>COX-2−/−</td>
<td>Vehicle</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>6†</td>
<td>Wild-type</td>
<td>SC-560</td>
<td>2 × 1.25</td>
</tr>
<tr>
<td>5</td>
<td>6†</td>
<td>Wild-type</td>
<td>Celecoxib</td>
<td>1 × 25</td>
</tr>
<tr>
<td>6</td>
<td>6†</td>
<td>COX-1−/−</td>
<td>SC-560</td>
<td>2 × 1.25</td>
</tr>
<tr>
<td>7</td>
<td>24†</td>
<td>Wild-type</td>
<td>Rofecoxib</td>
<td>1 × 5</td>
</tr>
<tr>
<td>8</td>
<td>10†</td>
<td>Wild-type</td>
<td>Valdecoxib</td>
<td>1 × 5</td>
</tr>
<tr>
<td>9</td>
<td>12†</td>
<td>COX-1−/−</td>
<td>Rofecoxib</td>
<td>1 × 5</td>
</tr>
<tr>
<td>10</td>
<td>12†</td>
<td>COX-2−/−</td>
<td>SC-560</td>
<td>2 × 5</td>
</tr>
<tr>
<td>11</td>
<td>10†</td>
<td>Wild-type</td>
<td>Piroxicam</td>
<td>1 × 5</td>
</tr>
<tr>
<td>12</td>
<td>24†</td>
<td>Wild-type</td>
<td>SC-560  + Rofecoxib</td>
<td>1 × 5</td>
</tr>
</tbody>
</table>

Animals were treated for 1, 4, 7, and 10 days (*), for 4 and 7 days (†), and for 7 days (‡), respectively.
The stomach was removed and stretched on a paraffin panel. The ulcer crater and a millimeter paper were photographed, and the pictures were transferred to a personal computer. The ulcer area was measured as described (25). Serial parafin sections containing the ulcer region were cut (3-μm thick) and stained with hematoxylin and eosin (23, 25). Epithelial cell proliferation was quantified on day 4 as described (23–25). Briefly, 100 mg/kg BrdU were administered intraperitoneally 1 h before the stomach was removed. Paraffin tissue sections were stained with MAb against BrdU, and bound antibodies were visualized using antimouse biotin antibodies and streptavidin-coupled horseradish peroxidase as described (23–25). The percentage of BrdU-labeled cells was determined in the intact mucosa and in a 600-μm-wide ulcer margin (24). The percentage of labeled cell nuclei over the total number of counted nuclei was calculated. Angiogenesis was assessed by counting the number of microvessels immunostained in the ulcer bed with von Willebrand factor antibody as described (23, 25). The microvessel density was expressed as the number of microvessels per square millimeter of granulation tissue section. The length of the mucosal ulcer margin was measured in the center of the ulcer, and its width was defined as the distance between the ulcer crater and the border of the ulcer margin and the intact mucosa (23, 25). The thickness of the ulcer bed was measured below the ulcer margin on both sides and at the center of the ulcer crater, and the mean was calculated (23, 25). All sections were coded, and one investigator, who was unaware of the code, evaluated them. Six sections per mouse and staining were evaluated (×400 microscopic magnification).

**RT-PCR.** Four days after ulcer induction, samples from the intact anterior and ulcerated posterior gastric corpus wall in groups 1–5 were immediately frozen in liquid nitrogen. RNA samples were extracted from the tissue using the TRizol reagent. After total RNA extraction, all samples were tested for RNA integrity by electrophoresis and cDNA synthesis was performed (21). Primers were designed using the Primer Express program (Applied Biosystems). All primers produced single-amplication products. The experiments were performed using a GenAmp 5700 Sequence Detection system (Applied Biosystems, CA) in triplicate. A total of at least 15 data points was generated from each genotype and animal group. Expression ratios were calculated on the basis of primer efficiencies (E) and the crossing point deviation of gene expression relative to 18s. Primers used were:

\[
\text{GAC-3}^{5'} \text{H11032} \]

for NOS-3 (155 bp, E: 1.70),

\[
\text{AGAAGCTTTGAC-3}^{5'} \text{H11032} \]

for NOS-2 (123 bp, E: 1.93),

\[
\text{ACTGAC for NOS-1 (78 bp, E: 1.70),} \]

and

\[
\text{GGGCGCTGGGTGCTGA-3}^{5'} \text{H11032} \]

- GGCGATTGGTGGAGGT-3

for 18s (91 bp, E: 10(1/slope)).

**Prostaglandin synthesis and NOS activity in intact tissue.** The synthesis of 6-keto-PGF\(_1\alpha\) was measured 1 h after dosing in the intact gastric corpus wall on day 4. Gastric tissue (~30 mg) was incubated in 0.6 ml of oxygenated Tyrode solution at 37°C for 30 min. The release of 6-keto-PGF\(_1\alpha\) into the medium was determined using the radioimmunoassay as described (23).

Four days after ulcer induction, NOS activity was assessed 1 h after dosing in the intact anterior wall in groups 1–5. The tissue samples (~30 mg) were homogenized for 20 s in 200 μl ice-cold homogenization buffer as described previously (17). After centrifugation at 16,000 g for 30 min at 4°C, NOS activity in the supernatants was determined by monitoring the conversion of l-[\(^3H\)]arginine to the NO coproduct l-[\(^3H\)]citrulline as described previously (18). Briefly, a mixture of 20 μl of the supernatant and 180 μl of incubation buffer (for details, see Ref. 18) was incubated for 30 min at 37°C. The incubation mixture contained 25 μM l-arginine and 0.5 μCi l-[\(^3H\)]arginine. EDTA (1 mM) was used to determine inducible NOS activity, and N\(^\text{3}\)-nitro-l-arginine methyl ester (3 mM) was used to assess the inhibition of NOS activity. After 1 ml ice-cold 20 mM sodium acetate buffer (pH 5.0) containing 2 mM EDTA and 1 mM l-citrulline was added, the mixture was passed through a cation exchange chromatography column (0.5–0.6 g of Dowex AG50WX-8 resins; sodium form, 200–400 mesh size). l-[\(^3H\)]citrulline was separated from l-[\(^3H\)]arginine by elution with H₂O (3 ml) and quantified using a liquid scintillation analyzer (Packard 1600 CA). Results were expressed as nanomoles of l-[\(^3H\)]citrulline formed per minute per gram of protein. Protein was measured using a protein assay kit (Bio-Rad).

**Statistical analysis.** All data are presented as means ± SE. Comparison among experimental groups was performed using ANOVA followed by the Student’s t-test. P values <0.05 were regarded as significant.

**RESULTS**

**Gastric ulcer healing curve.** Macroscopic ulcer areas in wild-type mice were 3.92, 2.25, and 0.85 mm\(^2\) on days 1, 4, and 7, respectively (Fig. 1). The different treatment groups are shown in Table 1. Ulcer formation was identical in wild-type, COX-1–/–, and COX-2–/– mice (Fig. 1A). Gastric ulcer healing was not delayed in COX-1–/– and SC-560-treated (2 × 5 mg/kg) mice. In contrast, gastric ulcer healing was significantly delayed in COX-2–/–, celecoxib-treated (1 × 25 mg/kg), and rofecoxib-treated (1 × 5 mg/kg) mice with increased ulcer sizes on days 7 and 10 (Fig. 1). Dual COX inhibition with SC-560 + rofecoxib resulted in a severe delay in healing, with significant differences on days 7 and 10 compared with rofecoxib-treated mice. There were no postoperative complica-
The percentage of BrdU-labeled cells in the mucosal ulcer margin and the microvessel density in the ulcer bed were decreased in COX-2−/− and celecoxib-treated mice (Figs. 2C and 3, A and B). Furthermore, the ulcer area on day 7 and the thickness of the granulation tissue in the ulcer bed were increased, and the lengths of the ulcer margin were decreased in COX-2−/− and celecoxib-, rofecoxib-, and valdecoxib-treated mice (Fig. 4). In contrast, these parameters showed no significant differences among vehicle-treated wild-type, COX-1−/−, and SC-560-treated wild-type mice (Figs. 3 and 4).

*Healing parameters in combined COX-1 and COX-2 impairment.* SC-560 + rofecoxib-treated and piroxicam-treated mice as well as rofecoxib-treated COX-1−/− and SC-560-treated COX-2−/− mice showed a severe delay in ulcer healing, with significantly increased ulcer sizes compared with COX-2−/− mice (P < 0.05) and wild-type mice (P < 0.001; Fig. 4A). Furthermore, histological analyses showed strongly decreased lengths of the ulcer margin (Fig. 4B) and increased thickness of the ulcer bed (Fig. 4C) compared with both COX-2−/− and wild-type mice.

**Gastric 6-keto-PGF₁α synthesis in intact tissue.** Compared with vehicle-treated wild-type mice, gastric 6-keto-PGF₁α synthesis was decreased by >99% in COX-1−/− mice but unchanged in COX-2−/− and in celecoxib- (1 × 25 mg/kg) and rofecoxib-treated (1 × 5 mg/kg) wild-type mice. SC-560 dose dependently inhibited gastric 6-keto-PGF₁α synthesis by 62%, 89%, 93%, and 95% at daily doses of 2 × 1.25, 2 × 5, 2 × 12.5, and 2 × 37.5 mg/kg, respectively (Fig. 5A).

**Effect of different doses of SC-560 on gastric ulcer healing.** The dose-response curve for the effect of SC-560 on gastric ulcer area in wild-type and COX-1−/− mice on day 7 showed no significant effects at daily intragastric doses of 2 × 1.25, 2 × 5, and 2 × 12.5 mg/kg but significantly increased ulcer sizes at daily doses of 2 × 37.5 mg/kg both in wild-type and COX-1−/− mice. Because the molecular target of SC-560 is lacking in COX-1−/− mice, these findings suggest that the specificity of SC-560 for inhibiting COX-1 disappears at a daily dose of 2 × 37.5 mg/kg, causing COX-1-unrelated delay of ulcer healing (Fig. 5B).
COX and NOS mRNA expression by RT-PCR in wild-type mice. Expression of mRNA was assessed by RT-PCR in wild-type mice on day 4 and related to the globally expressed 18S reference gene. In the intact tissue, COX-1 mRNA levels were higher than COX-2 mRNA levels, and NOS-1 and NOS-3 mRNA levels were higher than NOS-2 mRNA levels (Fig. 6A). COX-2 and NOS-2 mRNA were upregulated in the repair tissue compared with the intact tissue by factors of 1.6 and 3.1, respectively. In contrast, COX-1 and NOS-3 were not significantly up- or downregulated and NOS-1 mRNA was downregulated in the repair tissue by a factor of 2.5 (Fig. 6B).

COX and NOS mRNA expression by RT-PCR in groups 1–5 in intact tissue. In the intact tissue on day 4, mRNA levels of COX-1, COX-2, NOS-1, and NOS-3 were comparable among groups 1–5, with no significant differences among the groups. NOS-2 mRNA levels were comparable among wild-type, COX-2/H11002/H11002/H11002 and SC-560-treated mice did not differ from those in wild-type mice. In contrast, COX-2/H11002/H11002/H11002 mice and celecoxib-, rofecoxib-, and valdecoxib-treated mice showed significantly increased ulcer areas, reduced lengths of the ulcer margin, and increased thickness of the ulcer bed compared with wild-type mice. Valdecoxib-, celecoxib-, and rofecoxib-treated mice showed comparable results to COX-2/H11002/H11002/mice. Rofecoxib-treated COX-1/H11002/H11002/H11002, SC-560-treated COX-2/H11002/H11002/H11002, and SC-560 + rofecoxib-treated mice showed larger ulcer areas and increased thickness of the ulcer bed than COX-2/H11002/H11002 mice. Rofecoxib-treated COX-2/H11002/H11002/H11002, SC-560-treated COX-2/H11002/H11002, and SC-560 + rofecoxib-treated mice showed reduced lengths of the ulcer margin compared with COX-2/H11002/H11002 mice. Values are means ± SE of at least 6 animals per group. *P < 0.01 vs. wild-type mice, **P < 0.05 vs. COX-2/H11002/H11002/H11002 mice and ***P < 0.001 vs. wild-type mice.
COX-2 groups with wild-type mice, the following results were found: Comparing upregulation in the repair tissue wild-type mice (Fig. 7).

Because upregulation in the repair tissue versus the intact tissue among the four COX-manipulated groups with wild-type mice, the following results were found: COX-1 mRNA was more upregulated (Fig. 8A) and vice versa, and COX-2 mRNA was more upregulated in COX-1−/− mice than wild-type ones by a factor of 2.5 (Fig. 8A) and vice versa, and COX-2 mRNA was more upregulated in COX-1−/− mice than wild-type ones by a factor of 4 (Fig. 8B). NOS-1 mRNA was comparably downregulated in the repair tissue (groups 1–5) (Fig. 8C). NOS-3 mRNA was significantly more upregulated compared with wild-type mice by factors of 1.6, 3.6, 5.9, and 1.8 in COX-1−/−, COX-2−/−, SC-560-treated (2 × 5 mg/kg), and celecoxib-treated (1 × 25 mg/kg) mice, respectively (Fig. 8D).

Because upregulation in the SC-560-treated mice occurred in both the intact and the repair tissue, upregulations of NOS-2 mRNA were described in the repair tissue comparing NOS-2 mRNA levels in relation to S18 levels. In the repair tissue on day 4, NOS-2 mRNA levels were increased compared with vehicle-treated wild-type mice by factors of 3.6, 4.6, 2.8, and 12.5 in COX-1−/−, COX-2−/−, SC-560-treated (2 × 5 mg/kg), and celecoxib-treated (1 × 25 mg/kg) mice, respectively (Fig. 7).

NOS activity in the intact tissue of groups 1–5. NOS activity was assessed by measuring the conversion of L-[3H]arginine to L-[3H]citrulline. SC-560-treated (2 × 5 mg/kg) mice showed a significant 183% increase in total NOS activity compared with wild-type mice, but NOS activity was unchanged in COX-1−/−, COX-2−/−, and celecoxib-treated mice (Fig. 9). In SC-560-treated mice, 53% of total NOS activity was NOS-2-mediated (calcium independent). In wild-type mice, NOS-2-mediated NO synthesis was low and below detection levels in several animals. As expected, NOS activity was profoundly inhibited in our control experiments by N\(^{\circ}\)-nitro-L-arginine methyl ester.

**DISCUSSION**

This study provides new molecular and functional information on the significance of COX and NOS isoforms during gastric ulcer healing. Whereas gene disruption and selective inhibition of COX-1 did not impair ulcer healing, gene disruption and selective inhibition of COX-2 moderately impaired ulcer healing. More severe healing impairment was found in the combined impairment of COX-1 and COX-2. Furthermore, different patterns of upregulation of COX and NOS isoforms were found in our COX-1 and COX-2 deficiency and inhibition mouse models.

Selective COX-2 inhibitors delay gastric ulcer healing by inhibiting COX-2-mediated PG synthesis in the repair tissue and by inhibiting epithelial cell proliferation and angiogenesis. Published studies on the deleterious effects of COX-2 inhibitors on gastric ulcer healing (19, 23) are limited by being unable to properly differentiate between COX-2-selective and COX-unrelated effects. In fact, all COX-2 inhibitors can affect epithelial and vascular repair mechanisms by interfering with a cascade of COX-dependent signaling pathways and affecting proliferation and apoptosis (27, 38). Our study used COX-2−/−,

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**Fig. 6.** Quantitative analysis of COX and nitric oxide synthase (NOS) mRNA expression by real-time RT-PCR in wild-type mice on day 4. A: relative mRNA expression was calculated and divided by the globally expressed 18S gene levels (target gene 1x/S18, in log 2 scale). In the intact tissue, COX-1 mRNA levels were higher (***P < 0.01) than COX-2 mRNA levels, and NOS-1 and NOS-3 mRNA were higher (**P < 0.01) than NOS-2 mRNA. B: expression ratios (log 2) showing up- or downregulation of COX and NOS isoforms for the repair vs. intact tissue were calculated (see MATERIALS AND METHODS). COX-2 and NOS-2 mRNA were upregulated (***P < 0.02), COX-1 and NOS-3 were not significantly up- or downregulated, and NOS-1 was downregulated (***P < 0.02) in the repair tissue compared with the intact tissue. Values are means ± SE of at least 5 animals per group.

**Fig. 7.** Quantitative analysis of NOS-2 mRNA expression by real-time RT-PCR in wild-type (group 1) and COX-manipulated mice (groups 2–5; see Table 1) on day 4. Relative NOS-2 mRNA levels were calculated and divided by the globally expressed 18S gene levels (target gene 1x/S18, in log 2 scale). NOS-2 mRNA levels were significantly higher in the repair tissue compared with the intact in groups 1–5 (for wild-type mice, see also Fig. 6). In the intact tissue, NOS-2 mRNA levels were significantly higher in SC-560-treated (2 × 5 mg/kg ig) compared with vehicle-treated mice. In the repair tissue, NOS-2 mRNA levels were significantly higher in all COX-manipulated mice (groups 2–5) compared with wild-type mice (group 1). Values are means ± SE of at least 5 animals per group.
mice to overcome this limitation and showed that the observed impairment of epithelial cell proliferation, angiogenesis, and healing delay is caused by the genetic lack of COX-2. Furthermore, we found a comparable delay in healing in COX-2/H11002/H11002 mice and celecoxib-, rofecoxib-, and valdecoxib-treated wild-type mice. Therefore, COX-2-specific mechanisms seem to be predominantly responsible for the delay in gastric ulcer healing in these four groups. Thus COX-2-derived PGs seem to be key mediators during gastric ulcer healing. This concept is also supported by previous ulcer healing studies in rats (31), which showed that gastric PG synthesis is about threefold higher in the repair tissue than the intact tissue and that these gastric PGs are predominantly mediated by COX-2 and, to a lesser extent, by COX-1 (31). These rodent studies have now been confirmed in human studies in which the COX-2 selective inhibitor celecoxib significantly delayed gastric ulcer healing (6).

In a previous ulcer healing study, the natural compound resveratrol was reported to be a selective COX-1 inhibitor that delays ulcer healing (4). In our studies, however, ulcer healing in COX-1/H11002/H11002 and SC-560-treated mice was unimpaired. Dose-response curves of the effect of SC-560 on ulcer healing in wild-type and COX-1/H11002/H11002 mice showed that SC-560 does not delay healing if COX-1 specific doses are administered. In contrast to the selective COX-1 inhibitor SC-560, resveratrol not only inhibits COX-1, but also impairs the induction and activity of COX-2 (30). Therefore, the observed resveratrol-induced healing delay seems to be due to the impairment of COX-1 and COX-2. On the basis of our data and previous ulcer formation studies (41), selective inhibition of COX-1 seems to be less damaging for the gastrointestinal tract than initially expected. Because gastric acid secretion profoundly affects gastric ulcer formation and healing (7, 13, 25), it is important to note that gastric acid secretion is maintained in COX-1/H11002/H11002 mice (2). COX-1/H11002/H11002 mice do not spontaneously develop gastric erosions despite a >99% decrease in gastric PG synthesis, and COX-1 selective inhibitors do not induce gastric lesions. Inhibition of both COX-1 and COX-2 was required for the formation of gastric lesions (41). These results are important because selective COX-1 inhibitors may be useful for chemoprophylaxis and antineoplastic therapy (42).

The role of COX isoforms has also been tested at other healing sites (16, 44). During bone healing, severe healing impairment was detected in COX-2/-/- but not in COX-1/-/-

Fig. 9. Total NOS activity was assessed by measuring the conversion of L-[3H]arginine to L-[3H]citrulline on day 4 in groups 1–5 (see Table 1). SC-560-treated mice (2 × 5 mg/kg ig) showed a 183% increase in total NOS activity compared with vehicle-treated wild-type mice. Values are means ± SE of at least 5 animals per group.
mice (44). In skin wound healing, the strength of the healing skin was substantially decreased in COX-2−/− mice as well as slightly decreased in COX-1−/− mice (16). Gastric ulcer healing, however, differs from other healing sites and is modified by parameters such as gastric acid secretion, gastric motility, and gastrointestinal growth factors.

It is now of clinical interest to assess whether selective COX-2 inhibition delays gastric ulcer healing less than unselective COX inhibition. We therefore performed several studies to assess whether COX-1-derived PGs become essential during healing if COX-2-derived PGs are deficient. Our studies showed that impairment of both COX-1 and COX-2 delays healing more than selective impairment of COX-2. Compared with COX-2−/− mice, more severe healing impairment was found in dual COX-1 and COX-2 inhibition (by SC-560 + rofecoxib), unselective COX inhibition (by piroxicam), combined COX impairment by gene disruption of COX-1 with COX-2 inhibition, and COX-2 gene disruption with COX-1 inhibition. Our new data support the concept that COX-1-derived PGs become important in gastric ulcer healing when COX-2-derived PGs are deficient and suggest that COX-2 selective inhibitors have the potential to be superior to traditional NSAIDs regarding healing impairment. This is an important finding because conclusive clinical studies cannot be performed in humans for ethical reasons.

In the intact gastric wall of wild-type mice, COX-1 mRNA levels were substantially higher than COX-2 mRNA levels and PG synthesis was, as expected, largely COX-1 mediated. Total gastric PG synthesis was substantially decreased in COX-1−/− and SC-560-treated mice and remained unchanged in COX-2−/− and celecoxib-treated mice. In the intact gastric wall, NOS-2 mRNA levels and NOS-2-mediated NO synthesis were low (17). Messenger RNA levels of NOS-1, NOS-2, and NOS-3 and total NOS activity showed no differences among COX-1−/−, COX-2−/−, and celecoxib-treated mice compared with wild-type mice (27). It is remarkable that a >99% decrease in total PG synthesis in COX-1−/− mice neither increased mRNA levels of NOS isoforms nor total NOS activity in these animals (27).

SC-560-treated mice showed increased NOS-2 mRNA levels and total NOS activity in the intact gastric wall. These results agree well with previous studies using different acute studies in rats, which showed that SC-560 as well as indomethacin decreased gastric blood flow (41), increased NOS-2 mRNA in the intestine (32), increased inducible NOS activity (32), increased gastrointestinal motility and mucosal permeability and bacterial invasion in the intestine, being a strong stimulator of NO generation and activity (32). In previous acute studies in rats (34), 30 mg/kg SC-560 caused an upregulation of COX-2 mRNA in the gastric mucosa 2–8 h after dosing. In our studies, 2 × 5 mg/kg SC-560 for 4 days did not cause a significant upregulation of COX-2 mRNA levels in the intact gastric mouse wall on day 4. The different results can be explained by differences in dosing, study time points (2–8 h vs. 4 days), tissue selection, and species. Upregulated COX-2 mRNA levels in the acute phase (2–8 h after dosing of SC-560) are likely to decrease and normalize in the subacute (day 4) and chronic phase due to adaptive mechanisms.

Compared with intact gastric tissue, COX-2 mRNA was upregulated in the ulcerated repair tissue of wild-type mice on day 4. As shown in previous studies, COX-2 protein became rapidly upregulated after ulcer induction (peak days 3–5) in the cytoplasm of fibroblasts, inflammatory cells, and endothelial cells in the ulcer bed (19, 23). COX-2-derived PGs initiate the early inflammatory phase of healing, promote epithelial cell proliferation and angiogenesis, and are important in the late remodeling phase of healing (23). Quantification of COX-1 mRNA levels showed high levels of COX-1 mRNA both in the intact and the repair tissue without significant differences between both tissues on day 4. Interestingly, COX-2 mRNA was more upregulated in the ulcerated repair tissue of COX-1−/− mice than in wild-type ones, and, vice versa, COX-1 mRNA was more upregulated in COX-2−/− mice than in wild-type ones. Comparable results were found during dermal wound healing in mice (16). This upregulation of the COX isoforms may be important to compensate for the lack of the other isoform during healing. These data also support our in vivo ulcer-healing studies, which showed a severe healing delay in combined COX-1- and COX-2-impaired mice.

Interestingly, SC-560- and celecoxib-treated mice did not show significantly increased levels of COX mRNA levels. The observed discrepancy between the results in the COX-deficient and COX inhibitor-treated mice needs to be explained. In contrast to COX-deficient mice, COX selective inhibitors only allow an incomplete inhibition of the COX isoform. This issue has been further studied in our experiments using different doses of SC-560. At the selected standard dose of 2 × 5 mg/kg SC-560, SC-560 inhibits COX-1-mediated gastric PG synthesis by 89% 1 h after dosing. At higher doses of SC-560 (2 × 37.5 mg/kg) with 95% inhibition of gastric PG synthesis, significant COX-1 unrelated side effects on gastric ulcer healing were detected and thus cannot be used for proper molecular studies of upregulation of COX isoforms.

NOS-1 mRNA was downregulated after ulcer induction and does not seem to play a relevant role in the early phase of healing. As expected, NOS-2 mRNA is rapidly upregulated after ulcer induction due to its expression in inflammatory cells (11, 17, 37). NOS-2-derived NO promotes vasodilation and vascular permeability during the early healing phase and helps to clear inflammatory cells in the later healing phase (37). NOS-3 mRNA becomes upregulated in the repair tissue predominantly due to its expression in endothelial cells in the ulcer bed (17). NOS-3-derived NO directly promotes angiogenesis and is essential for the angiogenic action of VEGF during healing (45). In our studies, upregulation of NOS-2 and NOS-3 mRNA in the ulcerated repair tissue in COX-manipulated mice was more pronounced than in wild-type mice. These new findings on the compensatory, increased upregulation of NOS-2 and NOS-3 mRNA may be important to limit the negative effects of gene disruption or inhibition of COX-1 or COX-2. In NSAID-impaired gastric ulcer healing with locally decreased PGs, NO seems to play an even more important role than during regular healing. These molecular results may explain earlier pharmacological studies that showed that NO-releasing NSAIDs interfere less with healing than traditional NSAIDs or even accelerate gastric wound healing (9).

In summary, COX-2 is a key mediator of gastric wound healing. Although COX-1 has no significant role in healing when COX-2 is unimpaired, it becomes important when it is impaired. Inhibition and gene disruption of COX-1 and COX-2 can cause a compensatory increase in the mRNA of COX and NOS isoforms in the repair tissue. Our results suggest distinc-
tive and important roles for the different isoforms of COX and NO in the healing of chronic gastric ulcers.

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