Nonsteroidal Anti-Inflammatory Drug-Activated Gene-1 Plays a Role in the Impairing Effects of Cyclooxygenase Inhibitors on Gastric Ulcer Healing

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ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) can impair gastric ulcer healing. This study investigates the involvement of NSAID-activated gene-1 (NAG-1) in ulcer repair impairment by cyclooxygenase (COX) inhibitors. Gastric ulcers were induced in rats by acetic acid. Four days later, animals received daily intragastric administration of the COX-2 inhibitor, 1 mg/kg, and valdecoxib (selective COX-2 inhibitor; 5 mg/kg), celecoxib (selective COX-2 inhibitor; 1 mg/kg), for 1, 3, or 7 days. Ulcerated tissues were processed to assess: 1) COX-1, COX-2, NAG-1, proliferating cell nuclear antigen (PCNA), and activated caspase-3 expression; 2) ulcer area; and 3) prostaglandin E2 (PGE2) levels. COX-1 expression was enhanced. Ulcer healing was delayed by indomethacin, DFU, and SC-560, but not by celecoxib and valdecoxib. Ulcer PGE2 levels were decreased by SC-560, DFU, celecoxib, valdecoxib, and indomethacin. NAG-1 was overexpressed in ulcerated tissues and further enhanced by indomethacin, DFU, and SC-560, but not by celecoxib or valdecoxib. PCNA expression in ulcerated areas was reduced by indomethacin, but not by the other test drugs. The expression of activated caspase-3 in ulcers was increased and enhanced further by indomethacin, DFU, and SC-560, but not by celecoxib and valdecoxib. These findings indicate that: 1) COX inhibitors exert differential impairing effects on gastric ulcer healing, through mechanisms unrelated to the inhibition of COX isoforms and prostaglandin production; and 2) NAG-1 induction, followed by activation of proapoptotic pathways, can contribute to the impairing effects of COX inhibitors on ulcer healing.

Introduction

Peptic ulcer is a tissue defect in the gastric or duodenal wall, which results from a necrotizing event and extends through the muscularis mucosae into the deeper layers. It is within these layers that the ulcerative process may erode a major blood vessel to elicit important bleeding (Yeomans and Naesdal, 2008). The main cause of peptic ulcer is represented by Helicobacter pylori infection, even though other factors are involved in its pathophysiology, including nonsteroidal anti-inflammatory drugs (NSAIDs) (Yeomans, 2011). Gastric acid secretion plays a pivotal role in the pathogenesis of peptic ulcer; therefore, ulcer therapy, in addition to H. pylori eradication, focuses on the inhibition of acid secretion (Yuan et al., 2006).

The clinical use of NSAIDs is associated with the occurrence of adverse effects in the upper digestive tract, such as...
gastric erosions, ulceration, bleeding, and perforation (Lanas et al., 2006; Scarpignato and Hunt, 2010). It is widely recognized that the detrimental effects exerted by NSAIDs on gastroduodenal mucosa depend on the blockade of cyclooxygenase (COX) isoenzymes (COX-1 and COX-2) and subsequent decrease in mucosal prostaglandin production (Musumba et al., 2009). However, there is increasing evidence to suggest that COX-independent topical mechanisms, such as the destruction of surface hydrophobic barrier and ion trapping followed by mitochondrial dysfunction, also contribute to the pathogenesis of gastric injury associated with NSAIDs (Lichtenberger, 2001; Scarpignato and Hunt, 2010).

NSAIDs are also able to impair the healing of pre-existing gastric ulcers (Halter et al., 2001; Ma et al., 2002; Schmassmann et al., 2006; Colucci et al., 2009), and here again both COX-dependent and COX-independent mechanisms seem to come into play. Current evidence suggests that NSAIDs exert their impairing effects on ulcer healing through the inhibition of COX isoforms. In particular, the inhibiting actions of NSAIDs on ulcer healing can be mimicked by COX-2-selective inhibitors (Ma et al., 2002; Schmassmann et al., 2006), thus supporting a significant involvement of COX-2 in ulcer repair. However, COX-1 could also play relevant roles, because the combined administration of selective COX-1 and COX-2 inhibitors was found to impair ulcer healing to a higher extent than selective COX-2 inhibitors alone (Schmassmann et al., 2006). Moreover, data regarding the expression of COX-1 and COX-2 in gastric ulcers are conflicting. Indeed, Jackson et al. (2000) found increased COX-2 immunostaining in macrophages, endothelial cells, and myofibroblasts with reduced epithelial expression at the ulcer edge, whereas COX-1 expression did not vary significantly in comparison with normal mucosa. By contrast, To et al. (2001) observed that at the ulcer edge COX-1 expression was increased in cells of the lamina propria, whereas COX-2 was strongly expressed in the hyperplastic foveolar epithelium, and that in the granulation tissue of the ulcer base there was a strong expression of COX-1 and COX-2 in myofibroblasts, macrophages, and endothelial cells. There is also evidence that COX-independent mechanisms could contribute to the impairing effects of NSAIDs on ulcer healing. For instance, the impairment of ulcer healing caused by COX-2 inhibitors endowed with N-substituted methansulphonamide structures, such as tert-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (L-745,337), was no longer observed with selective COX-2 blockers belonging to the 2,6-di-inden-5-yl-[2-(4-methylsulfonyl)phenyl]-2H-furanone (DFU; methylsulfonic, selective COX-2 inhibitor; 5 mg/kg/day), celecoxib (CEL; 4-[5-(4-methylphenyl)-3-(trifluoromethyl) pyrazol-1-yl]benzenesulfonyamide; sulfonamide selective COX-2 inhibitor; 1 mg/kg/day), or valdecoxib (VAL; 4-(5-methyl-3-phenylisoxazol-4-yl) benzenesulfonyamide; sulfonamide selective COX-2 inhibitor; 1 mg/kg/day). Sham ulcerated animals treated with drug vehicle were used as controls. The doses of test drugs were selected by means of preliminary in vivo experiments on the rat air pouch model of inflammation (Giere et al., 2005), as reported below. At days 1, 3, or 7 from the start of drug administration (5, 7, and 11 days from ulcer induction, respectively), the stomachs were rapidly removed, and their ulcerated areas were collected by cutting the tissue along the external edge of ulcer margin. Thus, each specimen consisted of a round, whole-thickness gastric tissue, which included the ulcer bed, the margin, and a small amount of the surrounding macroscopically normal mucosa. Histological assessments revealed that for each specimen the amount of normal tissue surrounding the ulcer margin did not exceed 10% of the whole specimen. The gastric specimens were processed for the assessment of the following parameters: 1) measurement of ulcer area; 2) assay of prostaglandin E2 (PGE2) levels; 3) immunochemical analysis of COX-1, COX-2, NAG-1, and cleaved caspase-3 and proliferating cell nuclear antigen (PCNA). The experimental design is summarized in Fig. 1. The experimental protocol was approved by the ethics committee for animal experimentation of the University of Pisa.

**Rat Air Pouch Model of Inflammation.** Test drugs were assayed on the in vivo rat air pouch model of inflammation as described previously (Giere et al., 2005) to select appropriate doses endowed with a potent and selective profile of inhibition on COX-1 and/or COX-2. Air pouches were produced by subcutaneous injection of 20-ml sterile air into the intrascapular area of the backs of male Sprague-Dawley rats (200–250 g; n = 6 for each group). Pouches

![Fig. 1. Diagram displaying the design of the study and the time course of experimental procedures.](image-url)
were allowed to develop for 1 day, during which animals were fasted with free access to water. COX inhibitors or vehicle were administered by the intragastric route 2 h before the injection of 2 ml of a 1% suspension of carrageenan dissolved in saline into the pouch. Three hours after carrageenan injection, the pouch fluid was collected by lavage with 1 ml of cold heparin saline. The fluid was centrifuged at 800g for 10 min at 4°C, and the supernatants were collected for analysis of PGE2 with an enzyme-linked immunosorbent assay kit (Cayman Chemical, Ann Arbor, MI). At the end of the 3-h postcarrageenan injection, stomachs were dissected, immediately frozen in liquid nitrogen, and stored at −80°C for subsequent PGE2 assay. The levels of PGE2 in the gastric mucosa were taken as an index of COX-1 activity, whereas those in the air pouch were considered as mirroring COX-2 activity (Gierse et al., 2005). The results were expressed as percentage of inhibition of PGE2 production versus control levels detected in animals treated with drug vehicle.

**Induction of Gastric Ulcer by Acetic Acid.** Gastric ulcers were induced as reported by Shigeta et al. (1998). Rats were anesthetized with chloral hydrate (300 mg/kg), and laparotomy was performed via a midline incision. After exposing the stomach, 40 μl of 20% acetic acid solution were injected into the subserosal layer of the border between the antrum and fundus on the anterior wall of the stomach, and the abdomen was then closed by suturing. Sham ulcerated rats were subjected to laparotomy without acetic acid injection. Four days after the induction of gastric ulcers, treatments with test drugs or their vehicles were started and repeated every 24 h for 1, 3, or 7 days. At those times, the animals were sacrificed, and the stomachs were rapidly removed and processed for subsequent assays.

The measurement of the ulcer area in stomachs excised from animals with acetic acid-induced damage was performed as described previously (Shigeta et al., 1998). In brief, the stomachs were rapidly removed, opened along the greater curvature, and pinned on a cork plate. Ulcer area (square millimeters) was then measured under a dissecting microscope provided with a millimetric grid by an operator who was unaware of the treatments given to the animals.

**Immunohistochemical Analysis of COX-1, COX-2, NAG-1, and Cleaved Caspase-3.** Specimens of gastric tissue, fixed in cold 4% neutral formalin diluted in phosphate-buffered saline (PBS), were dehydrated with ethanol, treated with xylene, and embedded in paraffin at 56°C. Serial sections (5 μm thick) were processed for immunostaining. Slides were treated with 1% hydrogen peroxide in methanol, microwaved in citrate buffer, and blocked with normal serum swine (1:20; DAKOPatts, Glostrup, Denmark). Sections were then incubated overnight at 4°C with the following primary antibodies: mouse anti-COX-1 and rabbit anti-COX-2 (1:150 and 1:100, respectively), rabbit anti-NAG-1 (1:4000), and rabbit anti-cleaved caspase-3 (1:75). Igs were diluted in PBS with 0.1% bovine serum albumin and 0.1% sodium azide. Sections were washed with PBS and incubated with biotinylated Igs followed by peroxidase-labeled streptavidin complex and 3,3′-diaminobenzidine tetrahydrochloride (DAB; DAKOPatts) (Fornai et al., 2006). Sections were counterstained with hematoxylin. All reactions were carried out at room temperature in a humidified chamber, and PBS was used for washes, unless otherwise specified. Specificity of immunopositive staining was assessed by preadsorbing primary antibodies with the respective blocking peptides at 10 times the antibody concentrations for 24 h at 4°C: COX-1 and COX-2 (Cayman Chemical) and cleaved caspase-3 (Asp175) (Cell Signaling Technology, Danvers, MA). Negative controls were obtained by substitution of the primary antibody with preimmune mouse/rabbit serum. Endogenous peroxidases and avidin-binding activity were assayed by incubating slides with DAB alone or peroxidase-labeled streptavidin complex/DAB, respectively. Staining intensity for NAG-1 and cleaved caspase-3 was evaluated by a semiquantitative method, according to which the immunoreactivity was graded as + + + when strongly positive, + + when positive, + when moderately positive, +/- when weakly positive, and 0 when negative. This evaluation was carried out by three independent observers for each selected gastric section.

**PGE2 Assay.** Enzyme immunoassay of PGE2 in the gastric mucosa was performed by using a commercial kit (Cayman Chemical), as described previously (Colucci et al., 2009). In brief, specimens of gastric mucosa were weighed, minced by forceps, and homogenized in 1 ml of cold phosphate buffer (0.1 M PBS, pH 7.4, containing 1 mM EDTA and 10 μM indomethacin) per gram of tissue by using a polytron homogenizer (QIAGEN, Milan, Italy). The resulting homogenate was added to an equal volume of absolute ethanol and stirred by vortex. After 5-min incubation at room temperature, the homogenate was centrifuged at 1500g for 10 min at 4°C. The supernatant was added with 1 N HCl until pH 4 was reached. Before performing the assay, samples were subjected to purification by using super-clean LC-18 SPE columns (Sigma, St. Louis, MO). For this purpose, 0.5 ml of sample was added to 2 ml of ethanol and vortexed. After incubation at room temperature for 5 min, the sample was centrifuged at 3000g for 10 min. The supernatant was then removed and applied to a LC-18 SPE column, previously activated with 5 ml of methanol followed by 5 ml of ultrapure water. The column was then washed with 5 ml of ultrapure water and 5 ml of hexane. PGE2 was eluted with 5 ml of ethyl acetate containing 1% methanol. The eluted ethyl acetate fractions were collected and evaporated to dryness under nitrogen. Aliquots were used for subsequent enzyme immunoassay. The results were expressed as picogram of PGE2 per milligram of wet tissue. The analysis of PGE2 in lavage fluid of air pouches was performed directly on the supernatant, obtained as reported above.

**Western Blot Assay of COX-1, COX-2, NAG-1, Cleaved Caspase-3, and PCNA.** Whole-thickness specimens of gastric ulcerated tissues, excised as reported above, were weighed and homogenized in lysis buffer containing: 10 mM HEPES, 30 mM NaCl, 0.2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 2% glycerol, 0.3 mM MgCl2, and 1% Triton X-100, using a polytron homogenizer (QIAGEN). Homogenates were spun by centrifugation at 15,000g for 15 min at 4°C, and the resulting supernatants were then separated from pellets and stored at −80°C. Protein concentration in each sample was determined by the Bradford method (Protein Assay Kit; Bio-Rad Laboratories, Hercules, CA). Thirty micrograms of total lysates were denatured by boiling, separated by 12% SDS-polyacrylamide gel electrophoresis, and then transferred onto Immobilon-P membrane. Blots were blocked and then probed with primary antibodies raised against rat COX-1, COX-2, NAG-1, uncleaved and cleaved caspase-3, PCNA and β-actin and detected by means of horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were visualized by incubation with chemiluminescent reagents and exposed to Kodak Image Station 440 (Carestream Health, Rochester, NY) for signal detection and densitometric image analysis.

**Reverse Transcription-Polymerase Chain Reaction of NAG-1 mRNA.** Expression of mRNA coding for NAG-1 was assessed by RT-PCR. At the time of extraction, samples of ulcerated gastric tissue, excised as reported above, were disrupted with cold glass pestles, and total RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA) and chloroform. Total RNA (1 μg) served as template for cDNA synthesis in a reaction using 2 μl of random hexamers (0.5 μg/μl) with 200 μl of Moloney murine leukemia virus reverse transcriptase in a buffer containing 500 μM deoxynucleoside-5′-triphosphate and 10 mM dithiothreitol. cDNA samples were subjected to PCR in the presence of primers based on cloned rat NAG-1 (sense primer, 5′-CCCCAGCTGTCGGGATCTC-3′; antisense primer, 5′-ATCATAGTCTGAGTACA-3′) (Böttner et al., 1999) and β-actin (sense primer, 5′-TTCATGAAAGTGACGCACTG-3′; antisense primer, 5′-CTTCAGAACGATTGGCCTGAGTAG-3′) (Fornai et al., 2006). PCR, consisting of 2 μl of RT products, 2.5 U of Taq polymerase, 100 μM deoxynucleoside-5′-triphosphate, and 0.5 μM primers, was carried out by a PCR thermocycler DNA Engine (Bio-Rad Laboratories). After 3 min at 94°C, the cycle conditions were 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C for 35 cycles, followed
by 7 min at 72°C. Aliquots of RNA not subjected to RT were included in PCRs to verify the absence of genomic DNA. The efficiency of RNA extraction, RT, and PCR was evaluated by primers for rat β-actin. PCR products (630 bp for NAG-1 and 286 bp for β-actin) were separated by 1.5% agarose gel electrophoresis in a Tris buffer (40 mM) containing 2 mM EDTA and 20 mM acetic acid, pH 8, and stained with ethidium bromide. PCR products were then visualized by UV light and subjected to densitometric analysis by the Kodak Image Station program (Carestream Health). The relative expression of target mRNA was normalized to that of β-actin.

**Drugs and Reagents.** The following drugs, antibodies, and reagents were used: indomethacin, acetic acid, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, sodium orthovanadate, and mouse anti-β-actin antibody (1:5000, Sigma); SC-560 and valdecoxib (Tocris Bioscience, Bristol, UK); DFU (Merck Research Labs, West Point, PA); celecoxib (kindly provided by Pfizer, Rome, Italy); mouse anti-COX-1 and rabbit anti-COX-2 (Cayman Chemical); rabbit anti-PCNA and horseradish peroxidase secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA); rabbit anticaspase-3 antibody (Cell Signaling Technology); and rabbit anti-NAG-1 (Millipore, Vigodrom, Italy). Other reagents were of analytical grade. Indomethacin, SC-560, DFU, celecoxib, and valdecoxib were suspended in 1% methocel and administered in a volume of 0.5 ml per rat.

**Statistical Analysis.** The results are presented as mean ± S.E.M. of values obtained from six to eight animals. The statistical significance of data were evaluated by one-way analysis of variance followed by post hoc analysis by Student-Newman-Keuls test, and significance of data were evaluated by one-way analysis of variance S.E.M. of values obtained from six to eight animals. The statistical expression of target mRNA was normalized to that of β-actin.

**Results**

**Assessment of COX Inhibition in the Air Pouch Model of Inflammation.** The evaluation of PGE$_2$ levels in the gastric mucosa and air pouch provided a quantitative assessment of the specificity of COX isoform blockade by test drugs in vivo (Gierse et al., 2005). Indomethacin dose-dependently decreased PGE$_2$ levels in both the gastric tissue and air pouch, with a maximal effect observed at 1 mg/kg. The degree of inhibition was similar for both COX isoforms (Table 1). The dose of 1 mg/kg/day has been previously shown to elicit a significant delay of gastric ulcer healing in rats without any relevant influence on animal mortality (Schmassmann et al., 1998; Shigeta et al., 1998). Therefore, the dose of 1 mg/kg/day was selected for subsequent experiments with indomethacin in the present study.

With regard to selective COX-1 or COX-2 inhibitors, the treatment of animals with SC-560 resulted in a reduction of gastric PGE$_2$ production, whereas air pouch levels were scarcely affected. A potent and selective blockade of COX-1 isoform was observed at 2.5 mg/kg (Table 1). Treatment with DFU, celecoxib, or valdecoxib evoked a significant reduction of air pouch PGE$_2$ levels in a dose-dependent fashion, whereas PGE$_2$ production in gastric tissue was only slightly decreased. A potent and selective inhibition of COX-2-derived PGE$_2$ was observed at 5, 1, and 1 mg/kg for DFU, celecoxib, and valdecoxib, respectively (Table 1). Overall, the doses of test drugs, which produced potent and selective inhibition of COX isoforms, were 2.5 mg/kg for SC-560, 5 mg/kg for DFU, and 1 mg/kg for celecoxib and valdecoxib. The doses of SC-560, DFU, and celecoxib were similar to those used previously for the evaluation of the effects of COX blockade on

### Table 1

Potency and selectivity of test drugs for COX isoforms in the air pouch model

<table>
<thead>
<tr>
<th>Drug</th>
<th>COX-1</th>
<th>COX-2</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Indomethacin</td>
<td></td>
<td></td>
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<tr>
<td>0.3 mg/kg</td>
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<td>59</td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>92</td>
<td>89</td>
</tr>
<tr>
<td>3 mg/kg</td>
<td>98</td>
<td>96</td>
</tr>
<tr>
<td>SC-560</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>82</td>
<td>8</td>
</tr>
<tr>
<td>2.5 mg/kg</td>
<td>95</td>
<td>11</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>98</td>
<td>24</td>
</tr>
<tr>
<td>DFU</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 mg/kg</td>
<td>2</td>
<td>55</td>
</tr>
<tr>
<td>5 mg/kg</td>
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<td>94</td>
</tr>
<tr>
<td>15 mg/kg</td>
<td>11</td>
<td>99</td>
</tr>
<tr>
<td>Celecoxib</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3 mg/kg</td>
<td>3</td>
<td>58</td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>14</td>
<td>91</td>
</tr>
<tr>
<td>3 mg/kg</td>
<td>17</td>
<td>99</td>
</tr>
<tr>
<td>Valdecoxib</td>
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</tr>
<tr>
<td>0.3 mg/kg</td>
<td>3</td>
<td>70</td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>8</td>
<td>95</td>
</tr>
<tr>
<td>3 mg/kg</td>
<td>16</td>
<td>94</td>
</tr>
</tbody>
</table>

*a* Inhibition of gastric PGE$_2$,  
*b* Inhibition of air pouch PGE$_2$.  

![Fig. 2. A](image_url) Western blot analysis of COX-1 and COX-2 in gastric mucosa from sham ulcerated (SU) rats or in ulcerated tissue from rats with acetic acid-induced gastric ulcer treated with drug vehicle for 1 day (control ulcer, CU). Animals were subjected to ulcer induction 4 days before the onset of vehicle administration. Each column represents the mean ± S.E.M. (vertical lines) of values obtained from five to six animals. *, $p < 0.05$, significant difference versus SU. B, immunohistochemical detection of COX-1 and COX-2 in gastric mucosa from sham ulcerated rats after 1 day from the onset of drug vehicle administration. The bottoms of fundic glands show a strong COX-1 immunostaining, localized mainly within chief cells, and only a small amount of COX-2 is found in different cell types. Bar, 100 μm.
gastric ulcer healing in rats (Gretzer et al., 2001; Berenguer et al., 2002; Hatazawa et al., 2007), whereas the dose of valdecoxib has been shown to produce a selective COX-2 inhibition in rat (Gierse et al., 2005; Ahmad et al., 2009). For these reasons, the above doses were used in our subsequent experimental procedures.

**Western Blot Analysis of COX-1 and COX-2.** In gastric samples obtained from sham ulcerated rats, Western blot analysis revealed the expression of both COX-1 and COX-2 protein (Fig. 2A). This expression pattern was similar to that observed in the stomach of intact rats (data not shown). After 1 day from starting drug vehicle administration, the expression of COX-1 in ulcerated tissue decreased, whereas COX-2 expression was increased (Fig. 2A).

**Immunohistochemical Analysis of COX-1 and COX-2.** After 1, 3, or 7 days from the onset of drug vehicle administration (5, 7, and 11 days from surgery, respectively), sham ulcerated rats showed a morphologically normal gastric wall with considerable expression of COX-1 in the mucosal layer, particularly within epithelial cells of the neck and bottom region of fundic glands. The surface mucus-producing epithelium and muscularis propria were negative for COX-1 (Figs. 2B and 3A). In control ulcerated rats, treated with drug vehicle for 1 day, the gastric wall showed a clear mucosal/submucosal necrotic damage: polymorphonuclear exudates were evident at the ulcer base, and inflammatory infiltrates occurred within the lamina propria and submucosa of ulcer margins. COX-1 immunostaining was significantly reduced at both the ulcer margin and bed, and such a pattern was maintained throughout the study period, although a partial recovery was detected at the ulcer margin on days 3 and 7. Strong COX-1 expression was appreciable in most of the epithelial glandular cells just around the ulcer margins (Fig. 3A). With regard to COX-2 immunoreactivity, a low amount of constitutive COX-2 was observed in the gastric mucosal layer of sham ulcerated rats at days 1, 3, or 7 from the onset of drug vehicle administration. In particular, COX-2 expression was detected in epithelial cells at the gland bottoms and in stromal cells surrounding the glands (Figs. 2B and 3B). In the gastric wall of control ulcerated rats treated with drug vehicle for 1 day the pattern of COX-2 expression was markedly enhanced: the ulcer margin and surrounding mucosa displayed increased COX-2 immunostaining at the level of the epithelial glandular and stromal cells (Fig. 3B). COX-2

![Image](image_url)

**Fig. 3.** Immunohistochemical detection of COX-1 (A) and COX-2 (B) in sham ulcerated rats or ulcerated tissue from rats with acetic acid-induced gastric ulcer after 1, 3, and 7 days from the onset of drug vehicle administration (5, 7, and 11 days from surgery, respectively). A, a considerable amount of COX-1 was present in the normal gastric mucosa of sham ulcerated rats, particularly in the neck and base of fundic glands. COX-1 expression was switched off in the ulcerated mucosa at day 1, with the exception of the mucosa just around the ulcer margin and the ulcer bed. COX-1 expression partly recovered at ulcer margins on subsequent days. B, a little COX-2 amount was constitutively present in the gastric mucosa from sham ulcerated rats, mainly in gland bottom epithelial cells. At day 1 the ulcerated gastric wall displayed a marked enhancement of COX-2 expression in the mucosa of ulcer margin and bed, where many spindle-shaped mesenchymal cells were present. This expression pattern was progressively attenuated on subsequent days, with the exception of COX-2 immunostained stromal cells at the ulcer bed. Bars, 100 μm.
was also strongly expressed in the granulation tissue beneath the ulcer bed, particularly in stromal, spindle-shaped cells displaying the morphological features of fibroblasts/myofibroblasts, as appreciable at higher magnification. At days 3 and 7 from the onset of drug vehicle administration the pattern of COX-2 expression was progressively reduced along the ulcer margin, although COX-2-immunostained spindle-shaped cells remained evident at the ulcer bed (Fig. 3B).

**Gastric Ulcer Healing.** The injection of acetic acid in the subserosal layer of the gastric wall caused the development of single macroscopic ulcers, which were evident after 5, 7, and 11 days (1, 3, and 7 days from the onset of drug treatment, respectively). In control animals, the ulcer area evaluated at days 1, 3, and 7 of drug vehicle administration accounted for 59.3 ± 4.3, 57.4 ± 3.8, and 20.2 ± 2.9 mm², respectively (Fig. 4A). At day 1 of drug administration none of the COX inhibitors were able to significantly affect the ulcer area compared with vehicle-treated control animals (Fig. 4A). At day 3 of drug treatment the ulcer area was significantly higher in animals receiving indomethacin (1 mg/kg/day) or DFU (5 mg/kg/day), whereas celecoxib (1 mg/kg/day), valdecoxib (1 mg/kg/day), or SC-560 (2.5 mg/kg/day) were without effects (Fig. 4A). Treatment of ulcerated rats with indomethacin, DFU, or SC-560 for 7 days was associated with a significant delay in gastric ulcer healing, whereas celecoxib and valdecoxib did not produce any significant effect (Fig. 4A).

**Assay of PGE₂.** Gastric tissue PGE₂ levels in sham ulcerated animals amounted to 547 ± 28.7 pg/mg tissue. This value did not differ significantly from that obtained for the stomach of intact animals (524 ± 21.3 pg/mg tissue). In ulcerated gastric tissues from control rats treated with drug vehicle for 1 day PGE₂ concentration was significantly increased (746 ± 35.8 pg/mg tissue) (Fig. 4B). At this time point, the administration of all test drugs was associated with a significant reduction of PGE₂ content in ulcerated tissues with a more pronounced effect observed after treatment with indomethacin (1 mg/kg/day) (Fig. 4B). Similar results were obtained at days 3 and 7 of drug administration (Fig. 4B).

**RT-PCR Analysis of NAG-1 mRNA.** In gastric tissues from sham ulcerated rats, RT-PCR analysis revealed the basal expression of NAG-1 mRNA (Fig. 5A). This expression pattern did not differ significantly from that observed in intact animals (data not shown). In control ulcerated rats receiving drug vehicle for 1 or 7 days NAG-1 expression was enhanced (Fig. 5A). Treatment of ulcerated animals with SC-560 (2.5 mg/kg/day), DFU (5 mg/kg/day), or indomethacin (1 mg/kg/day) for 1 day induced a significant enhancement of NAG-1 expression in the ulcerated tissue, whereas celecoxib (1 mg/kg/day) or valdecoxib (1 mg/kg/day) did not modify such an expression pattern (Fig. 5A).

**Western Blot Assay of NAG-1, Cleaved Caspase-3, and PCNA.** The expression of NAG-1 was detected in gastric tissue PGE₂ levels in sham ulcerated rats, RT-PCR analysis revealed the basal expression of NAG-1 mRNA (Fig. 5A). This expression pattern did not differ significantly from that observed in intact animals (data not shown). In control ulcerated rats receiving drug vehicle for 1 or 7 days NAG-1 expression was enhanced (Fig. 5A). Treatment of ulcerated animals with SC-560 (2.5 mg/kg/day), DFU (5 mg/kg/day), or indomethacin (1 mg/kg/day) for 1 day induced a significant enhancement of NAG-1 expression in the ulcerated tissue, whereas celecoxib (1 mg/kg/day) or valdecoxib (1 mg/kg/day) did not modify such an expression pattern (Fig. 5A).

**Western Blot Assay of NAG-1, Cleaved Caspase-3, and PCNA.** The expression of NAG-1 was detected in gastric
expression in the ulcerated tissue, whereas celecoxib (1 mg/kg/day) or valdecoxib (1 mg/kg/day) were without effect (Fig. 5B). It is noteworthy that in preliminary experiments we had observed that, when tested at the dose of 3 mg/kg/day, celecoxib or valdecoxib did not affect the expression of NAG-1 protein in gastric ulcerated tissues (data not shown).

The activation of caspase-3 was found to be increased in control gastric ulcerated tissues after 1 day of drug vehicle administration compared with sham ulcerated rats (Fig. 6A). Under these conditions, the administration of SC-560 (2.5 mg/kg/day), DFU (5 mg/kg/day), or indomethacin (1 mg/kg/day) for 1 day elicited a significant increase in NAG-1 expression in the ulcerated tissue, whereas celecoxib (1 mg/kg/day) or valdecoxib (1 mg/kg/day) did not exert any significant effect (Fig. 6B).

The expression of PCNA was not increased in gastric ulcerated tissues after 1 day of treatment with drug vehicle compared with sham ulcerated rats (Fig. 6C). Under these conditions SC-560 (2.5 mg/kg/day), DFU (5 mg/kg/day), celecoxib (1 mg/kg/day), and valdecoxib (1 mg/kg/day) did not modify the expression of PCNA, whereas treatment with indomethacin (1 mg/kg/day) was associated with a significant decrease (Fig. 6D).

**Immunohistochemical Analysis of NAG-1 and Cleaved Caspase-3.** The expression of NAG-1 and cleaved caspase-3 was detected in gastric wall samples from sham ulcerated and control ulcerated rats after 1 day from the start of drug vehicle administration (Fig. 7). With regard to NAG-1 expression, the gastric mucosa of sham ulcerated rats showed immunostained epithelial cells at both the surface and gland bottom. In control ulcerated rats, NAG-1 expression was full-thickness enhanced at both the ulcer margin and bed. A specific staining was appreciable in most of the mucous-producing and glandular cells of ulcer margin; specific granular immunoprecipitates also were found in the smooth muscle cells of vessels and muscularis propria. Considerable amounts of NAG-1 immunoreactive infiltrating cells, spindle-shaped cells, and smooth muscle cells were evident at the ulcer bed. Cleaved caspase-3 was found to be constitutively expressed in the surface epithelium and mucous neck cells of sham ulcerated rats. This expression pattern was enhanced in control ulcerated rats. In particular, marked staining was observed in surface epithelial cells at the ulcer margins as well as in infiltrating cells and spindle-shaped mesenchimal cells of the granulation tissue at the ulcer bed (Fig. 7). When animals were treated with test drugs for 1 day, SC-560 (2.5 mg/kg/day), DFU (5 mg/kg/day), and indomethacin (1 mg/kg/day) were associated with a significant increment of both NAG-1 and cleaved caspase-3 expression in the ulcerated tissues, whereas celecoxib (1 mg/kg/day) or valdecoxib (1 mg/kg/day) did not exert any significant effect (Table 2).

**Discussion**

The present study examined the effects of different COX inhibitors on the healing of experimental ulcers in an attempt to unravel the possible contribution of mechanisms unrelated to COX inhibition. Our findings are consistent with the idea that: 1) COX-independent mechanisms take a prominent part in the delaying action of COX inhibitors on ulcer healing, depending on the chemical structure of individual drugs; and 2) NAG-1 induction plays a role in the mechanisms through which COX inhibitors delay ulcer healing.

In our experiments, the COX-1/COX-2 inhibitor indomethacin caused a marked delay in ulcer healing, in agreement with previous studies (Konturek et al., 2005; Hatazawa et al.,
Likewise, the COX-1 inhibitor SC-560 significantly impaired ulcer healing, as reported previously (Konturek et al., 2005). When considering the effects of celecoxib, we did not observe any effect. In keeping with our findings, Poonam et al. (2005) showed that the healing of chronic gastric ulcers was not affected by 14-day treatment with celecoxib in rats. It is noteworthy that in a study on healthy volunteers celecoxib, at the maximal daily dose of 800 mg/day, did not
impair the healing of gastric lesions induced by endoscopic biopsy forces (Dikman et al., 2009). However, literature data on celecoxib are conflicting. For instance, Tibble et al. (2001) found that celecoxib, administered for 6 days at 9 mg/kg/day, delayed ulcer healing in rats. Likewise, a 15-day treatment with celecoxib at 1.8 mg/kg twice daily impaired ulcer repair in rats (Berenguer et al., 2002). In those reports, the time course of drug administration differed from ours, because in both cases drug treatments were started 24 h after ulcer induction, whereas we allowed a full development of ulcer before starting the administration of test drugs. By contrast, Ma et al. (2002) followed the same administration schedule adopted in our study, but celecoxib, given at 10 mg/kg/day, was found to delay ulcer healing. When considering the dose issue, it is important to note that we took care of selecting appropriate doses of COX inhibitors, by performing preliminary experiments to identify the doses falling within the range of selectivity for COX isoforms. Such dose-finding experiments represent a point of strength in support of the findings yielded by our subsequent experiments, because, based on this strategy, we could observe that the COX-2 inhibitors celecoxib, valdecoxib, and DFU, once tested at doses sparing COX-1, exerted differential effects on ulcer healing, thus suggesting that some of them were likely to act by COX-2-independent mechanisms. In this regard, in comparison with our results, literature data support the view that increased doses and/or prolonged administration of COX-2 inhibitors may lead to ulcer healing delay, and therefore it is conceivable that a loss of COX-2 selectivity, consequent to the administration of celecoxib at high doses for prolonged periods, could explain its inhibitory effects on ulcer repair, as reported in previous studies (see Supplemental Data).

When evaluating the expression of COX isoforms in gastric ulcers, we observed a progressive reduction of COX-1 immunopositivity at days 1 and 3, followed by a recovery at day 7 of drug vehicle administration. By contrast, COX-2 expression increased at days 1 and 3 and recovered at day 7. We then moved on to assay PGE$_2$ in ulcer tissue specimens, because prostaglandins have been reported to play a role in ulcer repair (Konturek et al., 2005). In our experiments, ulcer induction was associated with an enhancement of PGE$_2$ levels, likely as a consequence of the enhanced COX-2 expression. In addition, all test drugs decreased PGE$_2$ levels, with magnitudes that were fairly consistent with their COX selectivity and the expression patterns of COX isoforms. However, the inhibitory profiles of our test drugs on PGE$_2$ levels did not correlate with their effects on ulcer healing. Previous studies have shown that the impairing actions of nonselective NSAIDs on ulcer healing can be shared by selective COX-2 inhibitors, suggesting a predominant role of COX-2 in ulcer repair (Shigeta et al., 1998; Halter et al., 2001). Nevertheless, current evidence on the expression of COX isoforms in gastric ulcers is conflicting, and a role in ulcer healing also has been proposed for COX-1 (Jackson et al., 2000; To et al., 2001; Schmassmann et al., 2006). Overall, our results, taken together with the inconsistency of previous findings, support the view that COX-dependent mechanisms do not seem to play a predominant role in the impairing actions of COX inhibitors on gastric ulcer healing.

The present results show that different COX inhibitors, endowed with different chemical structures, exerted different effects on ulcer healing, irrespective of their COX-1 and/or COX-2 selectivity. The ability of nonselective NSAIDs and selective COX-2 inhibitors to exert pharmacological effects unrelated to COX inhibition is widely recognized (Baek and Eling, 2006; Wang et al., 2011). Moreover, the contribution of COX-independent mechanisms to the pathogenesis of gastric injury associated with NSAID therapy is well known (Scarpignato and Hunt, 2010). However, to the best of our knowledge, this is the first study specifically designed to evaluate the contribution of COX-independent mechanisms in the impairment of ulcer healing evoked by COX inhibitors. In particular, we paid attention to the fact that NSAIDs have been found to induce NAG-1 (Baek and Eling, 2006). For instance, in colonic cancer cell lines NAG-1 expression increased upon exposure to several NSAIDs, along with an enhancement of apoptosis (Baek et al., 2001). In the present study, our test drugs were found to affect differentially NAG-1 expression, because indomethacin, DFU, and SC-560 enhanced its expression, whereas celecoxib and valdecoxib were without effect. These results strongly correlate with the effects of our test drugs on ulcer repair, suggesting an involvement of NAG-1-dependent mechanisms in the ulcer delaying effects of indomethacin, DFU, or SC-560. Consistent with this contention, various COX inhibitors, including indomethacin and SC-560, were found to enhance NAG-1 expression in cancer cell lines, whereas celecoxib was without effect (Baek and Eling, 2006; Yamaguchi et al., 2008).

Taking into account that gastric ulcer healing is a complex process, which involves cell proliferation and apoptosis (Sánchez-Fidalgo et al., 2004), we examined PCNA expression, a marker of cell proliferation, and caspase-3 activation, taken as an index of apoptotic cell death, in ulcerated tissues. In control ulcers there was an increase in caspase-3 activation, without appreciable variations of PCNA, which has been found to increase at later stages of ulcer healing (Fornai et al., 2009). Our results also showed that, among the test drugs, only indomethacin decreased PCNA expression, and treatment with SC-560, DFU, and indomethacin, but not celecoxib or valdecoxib, was associated with an increase in caspase-3 activation. It is noteworthy that the pattern of caspase-3 activation by test drugs did not reflect their profiles of COX-1/COX-2 selectivity, but rather their inducing.

### Table 2
Summary of NAG-1 and activated caspase-3 expression detected by immunohistochemistry in gastric tissues from sham ulcerated and control ulcerated rats after 1 day from starting vehicle or test drug administration.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Gastric Tissues</th>
<th>NAG-1</th>
<th>Caspase-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham ulcer</td>
<td>Mucosa</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>CU (drug vehicle)</td>
<td>UB</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>SC-560</td>
<td>UB</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>DFU</td>
<td>UB</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>UB</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Valdecoxib</td>
<td>UB</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>UB</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

CU, control ulcer; UB, ulcer bed; UM, ulcer margins.

Intensity of expression is reported as negative (−), weakly positive (+/−), moderately positive (+), positive (++), and strongly positive (+++).

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effects on NAG-1, suggesting that the impairing effects of SC-560, DFU, and indomethacin on ulcer healing were likely to be mediated by the activation of NAG-1-dependent proapoptotic signaling. In addition, immunohistochemical analysis revealed a rather high degree of tissue colocalization between NAG-1 and cleaved caspase-3 expression, thus strengthening further the concept of a correlation between NSAID-induced NAG-1 expression and apoptotic cell death. Differences in chemical structure might account for the ability of some COX inhibitors to induce NAG-1, trigger proapoptotic signals, and delay ulcer healing, irrespective of their selectivity for COX-1 and/or COX-2. For instance, DFU, a selective COX-2 inhibitor with methansulfonic structure, was found to delay ulcer healing and activate NAG-1 and caspase-3, whereas, by contrast, celecoxib and valdecoxib, both endowed with sulfonamidic structure, did not delay ulcer healing and did not affect NAG-1 expression or caspase-3 activation. This contention is supported by previous evidence that NSA-398 and L-745,337, both endowed with methansulfonic structures, were also found to delay chronic gastric ulcers (Lesch et al., 1998).

In conclusion, the present study provides the first demonstration that COX inhibitors differ significantly in their ability to impair gastric ulcer healing, and such a property depends on mechanisms unrelated to the inhibition of COX isoforms and prostaglandin production. Based on the present findings, it is suggested that NAG-1 induction, followed by the activation of proapoptotic pathways, contributes to the delaying effects of COX inhibitors on ulcer healing.

Authorship Contributions

Conducted experiments: Colucci, Antonioli, Ippolito, Segnani, Awwad, and Fornai.

Performed data analysis: Bernardini, Tuccori, and Blandizzi.

Wrote or contributed to the writing of the manuscript: Colucci, Bernardini, Blandizzi, Scarpignato, and Fornai.

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