NSAID-Induced Enteropathy: Are the Currently Available Selective COX-2 Inhibitors All the Same?

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ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) can induce intestinal mucosal damage, but the underlying mechanisms remain poorly understood. The present study investigated the effects of celecoxib, etoricoxib, indomethacin, and diclofenac on small bowel integrity in rats. Male rats were treated orally with test drugs for 14 days. Animals were processed for assessment of blood hemoglobin levels and hepatic mitochondrial functions, microscopic evaluation of small intestinal damage, Western blot analysis of cyclooxygenase-1 and -2 (COX-1, COX-2) expression, and assay of malondialdehyde (MDA), myeloperoxidase (MPO), and prostaglandin E2 (PGE$_2$) levels in small intestine. Indomethacin and diclofenac decreased blood hemoglobin levels, whereas etoricoxib and celecoxib were without effects. Celecoxib caused a lower degree of intestinal damage in comparison with the other test drugs. Indomethacin and diclofenac, but not etoricoxib or celecoxib, reduced intestinal PGE$_2$ levels. Test drugs did not modify intestinal COX-1 expression, although they enhanced COX-2, with the exception of celecoxib, which downregulated COX-2. Indomethacin, diclofenac, and etoricoxib altered mitochondrial respiratory parameters, although celecoxib was without effects. Indomethacin or diclofenac increased MDA and MPO levels in both jejunum and ileum. In the jejunum, etoricoxib or celecoxib did not modify such parameters, whereas in the ileum, etoricoxib, but not celecoxib, increased both MDA and MPO levels. These findings suggest that nonselective NSAIDs and etoricoxib can induce enteropathy through a topic action, whereas celecoxib lacks relevant detrimental actions. The selectivity profile of COX-1/COX-2 inhibition by test drugs and the related effects on prostaglandin production do not appear to play a major role in the pathogenesis of enteropathy.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are most commonly prescribed for their analgesic and anti-inflammatory properties. However, their use is associated with adverse events in the upper gastrointestinal (GI) tract, and it is being increasingly appreciated that these drugs can also exert detrimental effects on the lower GI tract, with potential serious outcomes (perforation, overt bleeding, and strictures) (Scarpignato and Hunt, 2010; Zeino et al., 2010). Although the lower GI toxicity of nonselective NSAIDs (ns-NSAIDs) is frequently underestimated, there is evidence that its prevalence may even exceed that recorded for the upper GI tract, with a frequency of life-threatening complications accounting for about one third of all ns-NSAID-dependent GI complications (Scarpignato, 2008; Zeino et al., 2010). The risk factors associated with NSAID-induced enteropathy have not been fully identified yet, but there is some evidence that they include aging, history of previous ns-NSAID-induced GI damage, and colonic diverticular disease (Ballinger, 2008; Bardou and Barkun, 2010; Lanas et al., 2012).

It has been suggested that the inhibition of cyclooxygenase isoenzymes (COX-1, COX-2) and the consequent reduction of endogenous prostanoids may not be sufficient to fully explain the alterations of intestinal mucosal integrity and permeability observed during treatments with ns-NSAIDs (Bjarnason and Takeuchi, 2009). Conversely, there is increasing evidence that a direct and prolonged contact of these drugs with the bowel mucosa plays a major role in the pathogenesis of NSAID-induced enteropathy and that the injuring potential of each NSAID is closely related to its acidic/lipophilic chemical structure (Bjarnason, 2013). Indeed, the peculiar chemical properties of most ns-NSAIDs allow them to penetrate into intestinal epithelial cells and give rise to massive intracellular accumulation. This increase in intracellular drug concentration then leads to inhibition of mitochondrial oxidative phosphorylation, with consequent ATP deficiency, followed by...
severe oxidative damage, marked dysfunction of tight intercellular junctions, and apoptotic cell death (Thiélin and Beaugerie, 2005; Scarpignato, 2008). These alterations result in increments of bowel mucosal permeability, with subsequent massive exposure to luminal aggressors (bacteria and their degradation products, bile acids, etc.) and onset of severe inflammatory reactions (Bjarnason and Takeuchi, 2009). In particular, endotoxin release, by bacteria translocated into the gut wall, promotes the production of proinflammatory cytokines (e.g., interleukin-1 and tumor necrosis factor [TNF]) and a marked activation of neutrophils, which, once having entered the bowel microcirculation, undergo a rapid activation leading to tissue invasion and massive production of detrimental reactive oxygen species (Zeino et al., 2010).

Interestingly, the type and prevalence of GI lesions in patients treated with NSAIDs appear to depend on the particular NSAID taken. In this respect, the introduction of selective COX-2 inhibitors (currently celecoxib and etoricoxib) into clinical use, as anti-inflammatory/analgesic drugs endowed with higher degrees of selectivity for COX-2 over COX-1 than ns-NSAIDs, has been associated with a significant reduction (~50%) in risk of upper GI lesions (Moore et al., 2006). Nevertheless, the intestinal toxicity of selective COX-2 inhibitors remains scarcely investigated, with conflicting evidence (Zeino et al., 2010). Clinical trials, conducted in the framework of the Multinational Etoricoxib and Diclofenac Arthritis Long-term (MEDAL) program, suggested that etoricoxib, a COX-2 inhibitor endowed with a low $pK_a$ value (Okumura et al., 2009), was associated with a similar prevalence of lower GI adverse events, compared with the ns-NSAID diclofenac (Laine et al., 2007). By contrast, as recently observed in the celecoxib vs omeprazole and diclofenac in patients with osteoarthritis and rheumatoid arthritis (CONDOR) trial (Chan et al., 2010), a 6-month treatment with celecoxib, a COX-2 inhibitor displaying a higher $pK_a$ value (Bjarnason et al., 2007), was associated with a lower incidence of clinically significant upper and lower GI events than that recorded in patients receiving diclofenac plus omeprazole.

Based on current clinical data, the favorable safety profile of celecoxib, at both upper and lower GI tract, might not reflect a selective COX-2 inhibitor class effect but rather result from its peculiar chemical properties (i.e., lack of acidic structure). However, direct comparative assessments of the effects of selective COX-2 inhibitors on lower GI tract are lacking. Accordingly, the present preclinical study was designed to evaluate the effects of celecoxib, in comparison with etoricoxib and the ns-NSAIDs indomethacin and diclofenac, on intestinal integrity in aged rats, with the aim of identifying the primary mechanisms involved in the pathophysiology of bowel mucosal damage.

**Materials and Methods**

**Animals, Drug Treatments, and Experimental Design.** Experiments were performed on aged (40-week-old) male Wistar rats (500–600 g). The animals were fed standard laboratory chow and tap water ad libitum and were not used for at least 1 week after their delivery to the laboratory. They were housed, three in a cage, in temperature-controlled rooms on a 12-hour light cycle at 22–24°C and 50–60% humidity.

Preliminary experiments were conducted with the purpose of identifying the timing and dose required to elicit lower GI injury. For this purpose, the effects of increasing doses of test drugs, administered by intragastric gavage twice daily, were assessed at day 7 and 14 of treatment: indomethacin [2-[1-[4-chlorophenyl]carboxyl]-5-methoxy-2-methyl-1H-indol-3-yl] acetic acid, 2, 3, and 4 mg/kg per day; diclofenac [2-[2-(2,6-dichlorophenyl)aminophenyl] acetic acid], 4, 8, and 12 mg/kg per day; etoricoxib [5-chloro-6'-methyl-3-[4-(methylsulfonyl)phenyl]-2,3'-bipyridine], 2.5, 5, and 10 mg/kg per day; and celecoxib [4-[5-(4-methylphenyl)-3(trifluoromethyl)pyrazol-1-yl)benzenesulfonamide], 2, 4, and 8 mg/kg per day. Indomethacin and diclofenac were tested either in the absence or in the presence of omeprazole ([R,S]-5-methoxy-2-[4-methoxy-3,5-dimethylpyridin-2-yl] methylsulfanyl)-1H-benzimidazol-2-yl) 0.7 mg/kg, administered once daily in the morning (Berenguer et al., 2002). When the optimal timing and dosing relationship for each NSAID and the occurrence of bowel injury were established, subsequent rat groups were treated for the shortest period and lowest dose level was identified as being harmful to the intestinal mucosa in preliminary experiments. At the same time, care was taken to ensure that the dose selected for test drugs fell within the range of their inhibitory activity for COX-1 and/or COX-2. For this purpose, all test drugs were assayed in the air pouch model of inflammation in rats (Colucci et al., 2012) (Supplemental Material). Preliminary data regarding body-weight variations and mortality rate are displayed in Supplemental Table 1. The results concerning the potency and selectivity of test drugs for COX isoforms in the air pouch model are displayed in Supplemental Table 2. Overall, based on our preliminary findings, the study experiments were carried out in accordance with the following treatment schedules:

- **Group 1:** drug vehicle (methocel 1%)
- **Group 2:** indomethacin 3 mg/kg per day
- **Group 3:** indomethacin 3 mg/kg per day + omeprazole 0.7 mg/kg per day
- **Group 4:** diclofenac 8 mg/kg per day
- **Group 5:** diclofenac 8 mg/kg per day + omeprazole 0.7 mg/kg per day
- **Group 6:** etoricoxib 5 mg/kg per day
- **Group 7:** celecoxib 4 mg/kg per day

Vehicle, indomethacin, diclofenac, etoricoxib, and celecoxib were administered twice daily (9:00 AM and 7:00 PM) by intragastric gavage in a volume of 300 μl of vehicle. Omeprazole was administered once daily in the morning by intragastric gavage in a volume of 300 μl of vehicle 1 hour before the dose of NSAID. A small volume of drug vehicle was used to minimize any possible interference with the effects of test drugs as a consequence of repeated daily administrations. Animal body weight was monitored twice weekly so that drug doses could be adjusted accordingly. Systolic blood pressure and heart rate were also assessed both 1 day before starting drug administrations (baseline) and before they were killed. Twenty-four hours after the last dose of test drugs, rats were anesthetized with chloral hydrate. Blood samples were collected from each animal for hemoglobin measurement. The whole GI tract was excised and samples were snap-frozen in liquid nitrogen and stored at –80°C for subsequent: analysis of mucosal prostaglandin E$_2$ (PGE$_2$) in the ileum, assay of malondialdehyde (MDA) and myeloperoxidase (MPO) levels in the jejunum and ileum, and Western blot analysis of COX-1 and COX-2 in the ileum. Other portions of tissues were fixed in 10% formalin for subsequent evaluation of microscopic GI damage. Liver was also removed for the assessment of mitochondrial function.

**Hemoglobin Analysis.** Hemoglobin analysis was performed on blood samples collected as reported above, by means of Quantichrom Hemoglobin Assay Kit (Bioassay Systems, Hayward, CA) and expressed as grams per deciliter.

**Microscopic Assessment of GI Damage.** The histologic evaluation of intestinal injury was carried out as previously described by Anthony et al. (1993). Upon removal, the whole intestinal tract was immediately injected with 10% formalin and left in the same fixative solution. After 30 minutes, it was opened along the antimesenteric border, cleaned of fecal content, and fixed in 10% formalin for 24 hours, dehydrated, cleared, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The slides were assessed for histologic changes, and a comparative analysis of the injury patterns was performed.
hours. Tissue samples were taken as described below to rule out any bias:

- The full length of the small intestine was measured.
- Proximal jejunum: Two specimens of 1.5–2 cm were taken 5 cm away from both the proximal and distal end of 37.5% of the small intestine total length.
- Distal jejunum: Two specimens of 1.5–2 cm were taken 5 cm away from both the proximal and distal end of 81% of the small intestine total length.
- Ileum: One centimeter of tissue proximal to the ileocecal valve was discarded and two specimens of 1.5–2 cm of ileum were taken at this edge as well as 5 cm away from the first ileum sample.

After fixation, tissues were embedded into paraffin blocks and were cut into consecutive serial 3-μm sections for staining with hematoxylin and eosin. Histologic damage was assessed by two observers, blind to the treatment, according to the score system proposed by Anthony et al. (1993) with minor changes. The intestinal damage was classified as reported in Table 1. Representative pictures, showing the histologic appearance of type 1, 2, and 3 lesions of jejunum and ileum, are displayed in Supplemental Fig. 1.

**Assay of Mucosal PGE2 Levels.** Enzyme immunoassay of PGE2 in the gastric and intestinal mucosa was performed using a commercial kit, as previously described (Fornai et al., 2005). In brief, specimens of mucosa were rapidly scraped from the underlying tissue layers, using two glass slides kept cold on ice. The mucosa was weighed, minced by forceps, and homogenized in 1 ml of cold phosphate buffer (phosphate-buffered saline, 0.1 M, pH 7.4, containing 1 mM EDTA and 10 μM indomethacin) per gram of tissue, using a Polytron homogenizer (Qiagen, Milan, Italy). The resulting homogenate was added to an equal volume of absolute ethanol and stirred by vortex. After a 5-minute incubation at room temperature, the homogenate was centrifuged at 1500g for 10 minutes at 4°C. The supernatant was treated with 1 N HCl until pH 4.0 was reached. Before performing the assay, the samples were subjected to purification using superclean LC-18 SPE columns (Sigma-Aldrich, 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 minutes, each sample was centrifuged at 3000g for 10 minutes. The supernatant was then removed and applied to the 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. PGE2 concentration was expressed as picograms per milligram of mucosal tissue. The analysis of PGE2 in lavage fluid of air pouches was performed directly on the supernatant, obtained as described in Supplemental Material.

**Western Blot Assay of COX-1 and COX-2.** Specimens of ileum, 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 minutes 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 8% 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, 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.

**Assessment of Mitochondrial Function.** Liver tissue was used to obtain the mitochondrial pellet by differential centrifugations (Calderone et al., 2010). Rat liver mitochondria were used for these studies, because purified healthy mitochondria from intestinal tissues could not be obtained in sufficient yields. However, it is accepted that there are no significant differences between mitochondria isolated from different organs in their response to uncoupling agents or inhibitors of the respiratory chain (Tyler, 1991; Diao et al., 2012). The liver mitochondrial suspension was then divided into two aliquots: one (2 mg/ml mitochondrial proteins) was used to evaluate the mitochondrial membrane potential, and the other one (0.8 mg/ml mitochondrial proteins) was used to characterize mitochondrial respiration by recording oxygen consumption.

Basal mitochondrial membrane potential (taken as an index of energetic reserve) and changes in membrane potential induced by well-known uncoupling agents, such as 2,4-dinitrophenol (DNP) and carbonyl cyanide m-chlorophenyl hydrazine (CCCP), were potentiometrically recorded by means of selective tetraphenylphosphonium (TPP⁺)-sensitive mini-electrodes coupled with a reference electrode (World Precision Instruments, Sarasota, FL), using data acquisition software (Biopac Systems Inc., Goleta, CA). In brief, before each experiment, electrodes were calibrated using known concentrations of TPP⁺ Cl⁻. Mitochondria (2 mg/ml protein) were suspended under gentle stirring in the incubation medium (composition: 120 mM KCl, 5 mM K₂HPO₄, 10 mM HEPES, 10 mM succinic acid, 2 mM MgCl₂, 1 mM EGTA, 10 μM TPP⁺ Cl⁻, pH 7.4 adjusted with KOH). The value of membrane potential was calculated according to the following Nernst-derived experimental equation:

\[ \Delta \psi = 60 \times \log \left( \frac{[TPP^+]_o}{[TPP^+]_i} \right) - V_0 - K_0 P \]

where \( \Delta \psi \) is the mitochondrial membrane potential (millivolts), \( V_0 \) is the volume of the incubation medium before mitochondria addition, \( V \) is the volume of the incubation medium after mitochondria addition, \( V_m \) is the volume of mitochondrial matrix (microliters per milligram protein), \([TPP^+]_o\) and \([TPP^+]_i\) are the concentrations of TPP⁺ recorded before the addition of mitochondria and at time \( t \), respectively, \( P \) is the protein concentration (milligrams per milliliter), \( K_0 \) and \( K_i \) are apparent external and internal partition coefficients of

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**TABLE 1**

<table>
<thead>
<tr>
<th>Microscopic criteria for the quantitative estimation of the intestinal injury elicited by test drugs</th>
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<tr>
<td><strong>Type 1 injury</strong></td>
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<tr>
<td><strong>Type 2 injury</strong></td>
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<tr>
<td><strong>Type 3 injury</strong></td>
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</tbody>
</table>

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**Table notes:**

- Damage confined to the tunica mucosa
- Significant morphologic alterations of villi
- Inflammatory infiltration in the submucosa, with thickening of the tunica muscularis or serosa
- The morphologic framework of the tunica mucosa is preserved
- Damage involves the full thickness of intestinal wall
- The morphologic patterns of tunicae are lost
- Inflammatory reaction widely extended to the tunica serosa with a significant increase in thickness
TPP\(^{+}\), and were estimated as 14.3 and 7.9 \(\mu\)g/mg, respectively. The volume of mitochondria was taken as 1 \(\mu\)l/mg protein (Calderone et al., 2010; Testai et al., 2013).

The efficiency of mitochondrial bioenergetic functions was evaluated by Clark oxygen electrode (World Precision Instruments) and acquired by means of the APOLLO 4000 Analyzer System (World Precision Instruments). In particular, the respiratory-control ratio (RCR, an index of the integrity of inner membrane), the mole of ATP synthesized per mole of oxygen consumed (ATP/O, an index of the efficacy of oxidative phosphorylation), and the “state III/UC ratio” (i.e., the ratio between respiring state and uncoupled state, a further index reflecting the extent of functional and physical integrity of the inner membrane) were calculated. In brief, mitochondria (0.8 mg/ml protein) were suspended under gentle stirring in the incubation medium (composition: 120 mM KCl, 5 mM K\(_2\)HPO\(_4\), 10 mM HEPES, 10 mM succinic acid, 2 mM MgCl\(_2\), 2 \(\mu\)M rotenone, pH 7.4 adjusted with KOH). Changes in oxygen concentration in the medium were continuously recorded by Clark electrode. Respiratory parameters were achieved using succinate as the substrate. When a stable basal respiration was recorded, 125 \(\mu\)M ADP was added to start state III (oxygen consumption rate when ADP is available), followed by state IV (oxygen consumption rate when ADP is exhausted). This allowed calculation of RCR, i.e., ratio state III/state IV. ATP/O was also calculated, i.e., the ratio of the moles of ATP produced over the relative moles of oxygen consumed. Finally, the uncoupling agent 100 \(\mu\)M DNP was added, to evaluate oxygen consumption under uncoupled condition, allowing calculation of the “state III/UC” ratio (Li and Graham, 2012).

**Evaluation of Tissue Oxidative Damage and Inflammatory Infiltrate.** MDA concentration in intestinal tissues was determined to obtain quantitative estimates of membrane lipid peroxidation (Fornai et al., 2005). For this purpose, intestinal tissue was excised, weighed, minced by forceps, homogenized in 2 ml of cold buffer (20 mM Tris-HCl, pH 7.4) using a Polytron homogenizer (Qiagen), and centrifuged at 1500\(g\) for 10 minutes at 4°C. Aliquots of supernatants were then used for subsequent assay procedures. Mucosal MDA concentrations were estimated using a colorimetric assay kit (Cayman Chemical, Ann Arbor, MI). Results were expressed as nanomoles of MDA per milligram of intestinal tissue.

MPO was assumed as a quantitative index to estimate the degree of intestinal wall infiltration by polymorphonuclear cells. Specimens of intestinal tissues (30 mg) were homogenized on ice with a Polytron homogenizer (Qiagen) in 0.6 ml of ice-cold lysis buffer (200 mM NaCl, 5 mM EDTA, 10 mM Tris, 10% glycerine, 1 mM phenylmethylsulfonyl fluoride, 1 \(\mu\)g/ml leupeptin, and 28 \(\mu\)g/ml aprotinin, pH 7.4). The homogenate was centrifuged two times at 4°C for 15 minutes at 1500\(g\). The supernatant was diluted 1.5 and used for determination of MPO concentration by means of enzyme-linked immunosorbent assay (ELISA) (Hycult Biotech, Uden, The Netherlands). The results were expressed as nanograms of MPO per milligram of intestinal tissue.

**Statistical Analysis.** The results are presented as mean ± SEM. The statistical significance of data was evaluated by one-way analysis of variance (ANOVA) followed by post hoc analysis by Student-Newman-Keuls test, and P values lower than 0.05 were considered significant. All statistical procedures were performed using GraphPad Prism 3.0 software (GraphPad Software, Inc., San Diego, CA). Linear regression analysis, performed by commercial software (GraphPad Prism), was used to analyze correlation between the expression of COX-2 (expressed as Log % of variation versus control) in animals treated with indomethacin, diclofenac, etoricoxib, and celecoxib. The choice of a semi-log analysis was justified by the observation that the distribution of MPO and MDA values was skewed, and therefore, their logarithmic conversion was performed to stabilize the variance (Howell, 2007).

**Results**

**Mortality.** In accordance with preliminary experiments (Supplemental Table 1), we expected some degree of mortality in rats treated with indomethacin alone (40%) or in combination with omeprazole (20%), as well as in the diclofenac group (20%). Therefore, in the main experiments, the number of rats included in these groups was increased, to obtain a minimum of 10 rats per group surviving until the completion of treatment period (Table 2).

**TABLE 2**

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Weight Variation</th>
<th>Number of Animals</th>
<th>Number of Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 7</td>
<td>Day 14</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>+10.0 ± 2.4</td>
<td>+22.5 ± 3.4</td>
<td>10</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>−10.6 ± 2.0*</td>
<td>−26.2 ± 3.1*</td>
<td>18</td>
</tr>
<tr>
<td>Indomethacin + omeprazole</td>
<td>−17.5 ± 5.9*</td>
<td>−28.0 ± 6.4*</td>
<td>14</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>−10 ± 4.6*</td>
<td>−39 ± 8.3*</td>
<td>14</td>
</tr>
<tr>
<td>Diclofenac + omeprazole</td>
<td>−17.5 ± 4.2*</td>
<td>−30.0 ± 8.0*</td>
<td>10</td>
</tr>
<tr>
<td>Etoricoxib</td>
<td>+8.3 ± 1.7</td>
<td>−5.0 ± 2.9*</td>
<td>10</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>−2.5 ± 2.5</td>
<td>−2.3 ± 2.1</td>
<td>10</td>
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*P < 0.05, significant difference vs. control.

**TABLE 3**

<table>
<thead>
<tr>
<th>Dose</th>
<th>Blood Pressure</th>
<th>Heart Rate</th>
<th>Hemoglobin</th>
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<tbody>
<tr>
<td>mg/kg per day</td>
<td>mm Hg</td>
<td>bpm</td>
<td>g/dl</td>
</tr>
<tr>
<td>Control</td>
<td>120 ± 3.5</td>
<td>350 ± 15</td>
<td>14 ± 0.3</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>3</td>
<td>115 ± 3</td>
<td>371 ± 17</td>
</tr>
<tr>
<td>Indomethacin + omeprazole</td>
<td>3</td>
<td>114 ± 5</td>
<td>372 ± 15</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>8</td>
<td>118 ± 3.8</td>
<td>352 ± 8.9</td>
</tr>
<tr>
<td>Diclofenac + omeprazole</td>
<td>8</td>
<td>116 ± 3</td>
<td>355 ± 11</td>
</tr>
<tr>
<td>Etoricoxib</td>
<td>5</td>
<td>125 ± 4.2</td>
<td>345 ± 11</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>4</td>
<td>121 ± 2.15</td>
<td>350 ± 12</td>
</tr>
</tbody>
</table>

*P < 0.05, significant difference vs. control.
Macroscopic Appearance of the Intestine. Given the large extension of small bowel surface, it was quite difficult to perform a reliable quantitative estimation of the macroscopic injury evoked by test drugs. However, qualitative inspection allowed documentation of the presence of macroscopic alterations, including, in particular, diaphragm-like strictures and multiple ulcerative lesions in animals treated with indomethacin or diclofenac, either in the absence or in the presence of omeprazole. By contrast, no appreciable macroscopic changes were detected in intestinal tissues obtained from rats treated with etoricoxib or celecoxib.

Assessment of Body Weight Variations. Animals treated with drug vehicle displayed an increase in body weight of $10 \pm 2.4$ and $22.5 \pm 3.4$ g at 7 and 14 days, respectively (Table 2). The administration of indomethacin (3 mg/kg per day), indomethacin plus omeprazole (0.7 mg/kg per day), diclofenac (8 mg/kg per day), or diclofenac plus omeprazole for 7 or 14 days was associated with a significant reduction of body weight. Animals treated with etoricoxib (5 mg/kg per day) displayed a significant reduction of body weight after 14 days of treatment, whereas the administration of celecoxib (4 mg/kg per day) was associated with a decrease in body weight at both 7 and 14 days, which however did not reach the statistical significance versus control (Table 2).

Systolic Blood Pressure and Heart Rate. Mean systolic blood pressure, recorded in control rats, was $120 \pm 3.5$ mm Hg, whereas heart rate was $350 \pm 15$ bpm (Table 3). In animals treated with indomethacin (3 mg/kg per day), indomethacin plus omeprazole (0.7 mg/kg per day), diclofenac (8 mg/kg per day), or diclofenac plus omeprazole for 14 days, no significant variations in both parameters were recorded (Table 3).

Blood Hemoglobin Analysis. The evaluation of blood hemoglobin levels was assumed as an indirect index of bleeding induced by test drugs in the GI tract (Sánchez et al., 2002). In control rats treated with drug vehicle for 14 days, hemoglobin concentration was $14 \pm 0.3$ g/dl (Table 3). Administration of indomethacin (3 mg/kg per day), indomethacin plus omeprazole (0.7 mg/kg per day), diclofenac (8 mg/kg per day), or diclofenac plus omeprazole for 14 days decreased blood hemoglobin levels. No significant changes were detected in animals treated with celecoxib (4 mg/kg per day), while animals treated with etoricoxib (5 mg/kg per day) tended to display reduced blood hemoglobin levels, although not significantly (Table 3).

Microscopic Evaluation of Intestinal Damage. Histologic analysis revealed that treatment with indomethacin (3 mg/kg per day), diclofenac (8 mg/kg per day), etoricoxib (5 mg/kg per day), or celecoxib (4 mg/kg per day) caused the occurrence of type 1 lesions (Fig. 1A). In the presence of omeprazole (0.7 mg/kg per day), indomethacin produced a lower degree of intestinal damage, whereas in rats treated with omeprazole plus diclofenac there were significantly higher degrees of damage as compared with diclofenac alone (Fig. 1A). Indomethacin and diclofenac evoked also the occurrence of type 2 and 3 lesions, whereas etoricoxib and celecoxib did not elicit any significant lesion (Fig. 1A). Indomethacin plus omeprazole reduced both type 2 and 3 lesions, whereas omeprazole caused a significant worsening of diclofenac-induced intestinal damage.

In the distal jejunum, all test drugs caused the occurrence of type 1 lesions (Fig. 1B). Treatment with indomethacin or diclofenac evoked also type 2 and 3 lesions, although no significant damage was observed in distal jejunum excised from rats treated with etoricoxib or celecoxib (Fig. 1B). Omeprazole significantly reduced type 2 and 3 lesions elicited by indomethacin, whereas the proton pump inhibitor enhanced the damaging actions of diclofenac (Fig. 1B).

In the ileum, all test drugs caused various degrees of type 1 lesions (Fig. 2). Treatments with indomethacin, diclofenac, etoricoxib, and, to a significantly lesser extent, celecoxib caused the occurrence of type 2 lesions. Omeprazole did not affect the degree of indomethacin-induced ileal type 2 lesions, while inducing a significant increase in the damaging effects evoked by diclofenac (Fig. 2). The occurrence of type 3 lesions

![Fig. 1. Effects of indomethacin (3 mg/kg per day), indomethacin plus omeprazole (0.7 mg/kg per day), diclofenac (8 mg/kg per day), diclofenac plus omeprazole, etoricoxib (5 mg/kg per day), or celecoxib (4 mg/kg per day) on microscopic damage in proximal (A) or distal jejunum (B). Each column represents the mean ± S.E.M. obtained from 10 to 11 animals. *P < 0.05, significant difference versus control; #P < 0.05, significant difference versus indomethacin; *P < 0.05, significant difference versus diclofenac.](image-url)
expression was increased in tissues obtained from rats effect the pattern of COX-1 expression. However, COX-2 protein (Fig. 3). Treatment with test drugs for 14 days did not analysis revealed the expression of both COX-1 and COX-2 testinal samples obtained from control rats, Western blot of mucosal PGE2 content in both regions of the small plus omeprazole for 14 days displayed a significant reduction (0.7 mg/kg per day), diclofenac (8 mg/kg per day), diclofenac in rat ileum was observed in animals treated with indomethacin, indomethacin plus omeprazole, diclofenac, diclofenac plus omeprazole, and, to a lesser extent, etoricoxib, whereas celecoxib did not evoke any significant type 3 lesion (Fig. 2).

**PGE2 Assay.** PGE2 levels in the mucosa of jejunum and ileum from control rats after 14 days of drug vehicle administration accounted for 148 ± 12 and 129 ± 10 ng/g tissue, respectively (Table 4). Animals treated with indomethacin (3 mg/kg per day), indomethacin plus omeprazole (0.7 mg/kg per day), diclofenac (8 mg/kg per day), or diclofenac plus omeprazole for 14 days displayed a significant reduction of mucosal PGE2 content in both regions of the small intestine, while etoricoxib (5 mg/kg per day) or celecoxib (4 mg/kg per day) did not evoke any significant effect (Fig. 2).

**Western Blot Analysis of COX-1 and COX-2.** In intestinal samples obtained from control rats, Western blot analysis revealed the expression of both COX-1 and COX-2 protein (Fig. 3). Treatment with test drugs for 14 days did not affect the pattern of COX-1 expression. However, COX-2 expression was increased in tissues obtained from rats treated with indomethacin (3 mg/kg per day), and, to a lesser extent, indomethacin plus omeprazole (0.7 mg/kg per day), diclofenac (8 mg/kg per day), diclofenac plus omeprazole, or etoricoxib (5 mg/kg per day), whereas celecoxib (4 mg/kg per day) reduced the pattern of COX-2 expression (Fig. 3).

**Assay of Mitochondrial Function.** None of the tested drugs administered for 14 days was able to affect the basal membrane potential recorded in hepatic mitochondria in comparison with controls (Supplemental Fig. 2). Hepatic mitochondria from rats treated with test drugs displayed no significant alterations in, ATP/O, or II/UC values, with the exception of etoricoxib, which slightly (albeit significantly) reduced the RCR index, suggesting a possible impairment of the integrity of both respiratory complexes and inner mitochondrial membrane (Supplemental Fig. 3).

When considering the reactivity to uncoupling agents (DNP and CCCP), indomethacin, indomethacin plus omeprazole, diclofenac, diclofenac plus omeprazole, or etoricoxib enhanced the sensitivity of mitochondria, while celecoxib did not exert any significant effect (Supplemental Fig. 4).

**Evaluation of Malondialdehyde Levels in Intestinal Tissues.** The levels of MDA in jejunal and ileal specimens obtained from control animals accounted for 17.3 ± 2.8 and 15.9 ± 1.5 nmol/mg of tissue (Fig. 4, A and B). The administration of indomethacin or diclofenac, either alone or in combination with omeprazole, increased MDA levels in both jejunum and ileum (Fig. 4, A and B). Etoricoxib or celecoxib did not elicit any significant change in MDA levels in jejunum, while in the ileum etoricoxib, but not celecoxib, elicited a significant increase in tissue oxidative stress (Fig. 4, A and B).

**Assay of Myeloperoxidase Levels in Intestinal Tissues.** In normal animals, MPO levels in jejunum and ileum were 6.7 and 3.0 ng/mg, respectively (Fig. 4, C and D). Treatment with indomethacin, diclofenac, or diclofenac plus omeprazole caused a significant increase in MPO contents in jejunum, whereas indomethacin plus omeprazole, etoricoxib, or celecoxib did not modify such parameter (Fig. 4C). In ileal tissues from rats treated with indomethacin, indomethacin plus omeprazole, diclofenac, diclofenac plus omeprazole, or etoricoxib there was a significant increase in MPO levels, while celecoxib did not produce significant variations (Fig. 4 D).

**Correlation Analysis.** Data obtained from COX-2 expression and levels of MPO or MDA in the ileum were analyzed by semi-log linear regression. In particular, in the y axis, COX-2 expression was given as percent change versus control value, while in the x axis, MPO or MDA were expressed as log of

### TABLE 4

<table>
<thead>
<tr>
<th>Treatment of 14 Days</th>
<th>Jejunum</th>
<th>Ileum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/kg per day</td>
<td>ng/g tissue</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>148 ± 12 129 ± 10</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>8</td>
<td>96 ± 11° 77 ± 9°</td>
</tr>
<tr>
<td>Indomethacin + omeprazole</td>
<td>8</td>
<td>93 ± 3° 79 ± 6°</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>5</td>
<td>101 ± 6° 68 ± 7°</td>
</tr>
<tr>
<td>Diclofenac + omeprazole</td>
<td>8</td>
<td>94 ± 4° 74 ± 5°</td>
</tr>
<tr>
<td>Etoricoxib</td>
<td>5</td>
<td>134 ± 7 130 ± 5</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>4</td>
<td>139 ± 8 131 ± 6</td>
</tr>
</tbody>
</table>

*P < 0.05, significant difference vs. control
percent changes versus the respective control values. Significant correlations between COX-2 expression with MPO ($r^2 = 0.944$, $P = 0.028$) or MDA ($r^2 = 0.924$, $P = 0.038$) were observed in animals treated with indomethacin, diclofenac, etoricoxib, and celecoxib (Fig. 5).

**Discussion**

The present study was conducted on aged rats, since ageing is a risk factor for NSAID-induced enteropathy (Lanas et al., 2012), even though elderly patients could display altered basal conditions as compared with the aged healthy animals employed in our experiments. Moreover, our model was set up to mirror clinical practice with NSAID chronic administration in humans, and indeed the present indomethacin regimen caused small bowel alterations, such as diaphragm-like strictures and multiple erosive lesions, together with a decrease in blood hemoglobin, known to occur in patients receiving chronic ns-NSAIDs (Nygaard et al., 1995; Maiden et al., 2005). Consistently, NSAID administration was found to affect the mitochondrial function, as previously observed in vitro by Somasundaram et al. (2000), even if indomethacin was able to hamper the electron transport along the respiratory chain in rat hepatic mitochondria when acutely applied in vitro. In our hands, this NSAID did not affect the basal membrane potential and respiratory parameters, but increased the sensitivity of mitochondria to uncoupling agents, likely as a consequence of the in vivo chronic treatment schedule.

As expected, treatment with diclofenac caused characteristic macroscopic alterations of small bowel and a decrease in blood hemoglobin levels. Consistently, the microscopic analysis showed that both indomethacin and diclofenac elicited intestinal injuries ranging from superficial type 1 to deeper type 3 lesions, although with differing degrees of severity. The evidence that both indomethacin- and diclofenac-induced enteropathies were associated with increments of small bowel oxidation, inflammatory infiltration, and mitochondrial dysfunctions, strongly suggests the involvement of a direct topic injury. However, these ns-NSAIDs decreased also the intestinal PGE$_2$ production, an effect not shared by celecoxib and etoricoxib. Therefore, the possibility that COX-1 blockade by indomethacin and diclofenac, with consequent reduction of prostaglandin production, could contribute also to enteropathy cannot be ruled out.

In the present setting, celecoxib was much safer, as compared with ns-NSAIDs. Animals treated with celecoxib did not display evident intestinal macroscopic alterations and/or reduction of blood hemoglobin. Moreover, celecoxib did not elicit type 3 deep lesions, while inducing a very low rate of type 2 lesions, and some type 1 erosions. Consistently, celecoxib did not increase bowel tissue oxidation, inflammation, and mitochondrial dysfunction, and it did not affect PGE$_2$ production. These observations agree with human data showing a good intestinal safety of celecoxib (Goldstein et al., 2005, 2007; Chan et al., 2010), and are consistent with the chemophysical and pharmacodynamic characteristics of celecoxib, which is characterized by a nonacidic high pH$_{50}$ value while selectively inhibiting COX-2 (Gierse et al., 2005). Other preclinical (Tibble et al., 2000; Menozzi et al., 2006) and clinical (Smecuol et al., 2001) studies have shown the inability of celecoxib to affect intestinal permeability, whose increment represents the key pathophysiological step leading to gross lesion formation (Bjarnason and Takeuchi, 2009). In addition, celecoxib was previously found to not alter mitochondrial oxidative phosphorylation (Tibble et al., 2000), which is regarded as a pivotal factor contributing to the disruption of the intestinal barrier, and thereby to the occurrence of NSAID-induced enteropathy.

In our hands, the enteric safety of etoricoxib was better than those of indomethacin and diclofenac, but less favorable as compared with celecoxib. In the etoricoxib-treated group, some animals developed macroscopic ulcerations. In addition, etoricoxib elicited both type 2 and 3 lesions, particularly in the ileum. However, these injuries were not associated with significant reductions of blood hemoglobin, an effect consistent with the lack of increase in fecal blood loss in humans (Hunt et al., 2003). With regard for the mechanisms underlying etoricoxib enteropathy, our data suggest a direct topic action. Indeed, ileal tissues from etoricoxib-treated animals displayed increased oxidation, inflammatory infiltration, and mitochondrial alterations without changes in local PGE$_2$ production. Notably, this drug was the only one able to affect the mitochondrial respiratory complexes and inner membrane integrity, as indicated by the RCR values, in addition to its ability to increase the mitochondrial sensitivity to uncoupling agents. Such an effect is likely to be unrelated to its selectivity profile, because, despite etoricoxib being more selective for COX-2 than celecoxib (Riendeau et al., 2001), in our experiments the latter drug was devoid of any effect on mitochondrial activity and did not increase the indexes of oxidative/inflammatory injury in intestinal tissues. Therefore, the differences in the enteric safety of etoricoxib and celecoxib can be explained considering that, at variance with celecoxib, etoricoxib is characterized by a low pH$_{50}$, similar to the majority of ns-NSAIDs (Paulson et al., 2001; Michaux and Charlier, 2004). It is also worth mentioning that this conclusion, based on the present preclinical findings, agree...
with the results of a clinical trial program (MEDAL) showing similar degrees of intestinal toxicity upon treatment with indomethacin or diclofenac (Laine et al., 2007).

Interesting conclusions can be drawn from data concerning the expression patterns of COX enzymes in the ileum, and the respective values of tissue PGE2 contents. In our model, both COX-1 and COX-2 were expressed in ileal tissues from control rats. COX-1 expression was not affected by any test drug. However, COX-2 expression was enhanced by indomethacin, diclofenac, or etoricoxib, while being decreased by celecoxib. It is noteworthy that the expression patterns of COX-2 were partly consistent with the damaging enteric actions of test drugs, and indeed a good correlation was found when the degrees of inflammatory injury (reflected by tissue MPO and MDA contents) associated with each test drug were compared with the respective changes in COX-2 expression. Overall, these observations support the view that changes in COX-2 expression at ileal level are likely to reflect the topical damaging activity of indomethacin, diclofenac, and etoricoxib, which caused significant degrees of bowel injury and elicited significant COX-2 induction (probably in the framework of the inflammatory response to topical damage), while celecoxib, which did not elicit bowel damage, did not enhance (and actually decreased) COX-2 expression. Although it has been stated that inhibition of both COX-1 and COX-2 is needed to induce small bowel injury (Takeuchi et al., 2010), the ability of etoricoxib to damage intestinal mucosa clearly shows that this is not the case. Moreover, studies in COX-1- and COX-2-deficient mice have suggested that the lack of COX-2 and the topical effect of NSAIDs (be they selective or not) lead to NSAID-enteropathy, without concomitant COX-1 inhibition (Hotz-Behofsits et al., 2010), and indeed, being devoid of topical irritancy, celecoxib was not enterotoxic, despite COX-2 inhibition.

In the present study, subsets of experiments were carried out in animals cotreated with indomethacin or diclofenac plus omeprazole, since these conditions have been adopted in clinical trials, where the effects of selective COX-2 inhibitors

Fig. 4. Malondialdehyde (A and B) and myeloperoxidase (C and D) levels in jejunum (upper panels) and ileum (lower panels) from rats treated with indomethacin (3 mg/kg per day), indomethacin plus omeprazole (0.7 mg/kg per day), diclofenac (8 mg/kg per day), diclofenac plus omeprazole, etoricoxib (5 mg/kg per day), or celecoxib (4 mg/kg per day). Each column represents the mean ± S.E.M. obtained from 10 to 11 animals. *P < 0.05, significant difference versus control.
Moreover, Watanabe et al. (2013) recently reported that PPIs exacerbate small bowel injury in patients with rheumatoid arthritis taking NSAIDs on long term. Overall, it appears that PPIs can exert differential effects on NSAID-induced enteropathy, depending on the NSAID and PPI considered, and even the experimental model and PPI regimen adopted. Thus, clear conclusions on the impact of PPIs on NSAID-induced enteropathy cannot be drawn and further investigations are needed to better address this issue.

In summary, the present investigation shows that in aged rats chronic administration of indomethacin induced the development of enteropathy, this model being likely more predictive of clinical practice than acute experimental settings. Under these conditions, ns-NSAIDs, characterized by low $pK_a$, can damage the small bowel primarily through a topic action on mucosal layer. Conversely from etoricoxib, a COX-2-selective inhibitor displaying low $pK_a$, celecoxib is unable to trigger topic damage to intestinal mucosa, and therefore it lacks significant detrimental actions on small bowel. Thus, the inhibition of COX-1 and/or COX-2 alone does not appear to trigger intestinal damage in the absence of topical injury, as also documented by the differential impact of celecoxib and etoricoxib on the indexes of oxidation, inflammation, and mitochondrial function.

Authorship Contributions

Participated in research design: Fornai, Calderone, Scarpignato, Blandizzi.

Conducted experiments: Fornai, Antonioli, Colucci, Pellegrini, Giustarini, Testai, Martelli, Matrangasi, Natale.

Contributed new reagents or analytic tools: Testai, Martelli, Natale, Calderone, Scarpignato.

Performed data analysis: Antonioli, Tuccori, Blandizzi.

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

References


were compared with those of NSAIDs plus proton pump inhibitors (PPIs) (Combe et al., 2009; Chan et al., 2010). Very interesting, and somehow intriguing, results were obtained when indomethacin or diclofenac were tested in combination with omeprazole. Although one would expect a lack of influence by PPIs on the injuring action of ns-NSAIDs on intestinal mucosa, different and opposing findings were obtained in our study: Omeprazole was apparently protective against indomethacin in the jejunum but not in the ileum (particularly in the case of type 2 and 3 lesions). However, when combined with diclofenac, omeprazole was found to markedly enhance the injuring action of diclofenac (both in the jejunum and ileum; particularly for type 2 and 3 lesions). Of note, both these conflicting observations are corroborated by literature data. Indeed, Kuroda et al. (2006) observed that lansoprazole counteracted small bowel injury in a rat model of indomethacin-induced enteropathy, whereas Wallace et al. (2011) and Satoh et al. (2012) found that some PPIs exacerbated the detrimental effects of naproxen or indomethacin on rat small bowel. In clinical settings, both Goldstein et al. (2005) and Hawkey et al. (2008) observed an increase in small intestinal lesions in healthy volunteers taking a combination of omeprazole and naproxen.

Fig. 5. Linear regression analysis between the expression of COX-2 and tissue levels of MPO (A) and MDA (B) in animals treated with indomethacin, diclofenac, etoricoxib, and celecoxib. In the y axis, COX-2 expression was given as percent change versus control value, while in the x axis, MPO or MDA were expressed as log of percent change versus the respective control values.
pantoprazole against NSAID-induced gastric mucosal damage. Nauyn Schmiedebergs Arch Pharmacol 372:79–87.


