ABSTRACT

Administration of selective and nonselective cyclooxygenase (COX)-2 inhibitors to rheumatoid arthritis patients taking low doses of acetylsalicylic acid (ASA) for cardiovascular prevention associates with increased risk of gastrointestinal bleeding. The present study was undertaken to investigate whether administration of HCT-3012 [(S)-6-methoxy-α-methyl-2-naphthaleneacetic acid 4-(nitrooxy)butyl ester], a nitric oxide (NO)-releasing derivative of naproxen, exacerbates gastric mucosal injury in arthritic rats administered low doses of ASA. Our results demonstrated that while treating arthritic rats with a dose of 30 mg/kg/day ASA causes detectable mucosal injury, but had no effect on arthritis score and interleukin-6 plasma levels, coadministration of naproxen and celecoxib (30 mg/kg/day), in combination with ASA from day 7 to day 21, attenuates arthritis development (P < 0.01 versus arthritis alone), but markedly enhanced gastric mucosal damage caused by ASA (P < 0.01 versus ASA alone). In contrast, coadministration of HCT-3012 (15 mg/kg/day) significantly attenuated arthritis development, because HCT-3012 was equally or more effective than naproxen and celecoxib in attenuating local and systemic inflammation (P > 0.001 versus arthritis) without exacerbating gastric mucosal injury caused by ASA. Arthritis development associates with gastric COX-2 induction, mRNA and protein, and enhanced gastric prostaglandin E2 (PGE2) synthesis (P < 0.01 versus control rats). Although all treatments, including celecoxib, were effective in reducing gastric PGE2 synthesis, administering arthritic rats with ASA resulted in a significant increase in gastric content of aspirin-triggered lipoxin (ATL), a COX-2-derived lipid mediator that regulates proinflammatory responses at the neutrophils/endothelial interface. Administering arthritic rats with naproxen and celecoxib abrogates ATL formation induced by ASA although enhanced neutrophils accumulate into the gastric mucosa (P < 0.01 versus ASA alone). In contrast, whereas HCT-3012 inhibited ATL formation, it did not increase neutrophil recruitment into the gastric microcirculation. Collectively, these data indicate that HCT-3012 derived from NO has the potential to compensate for inhibition of PGE2 and ATL and to protect the gastric mucosa by limiting the recruitment of neutrophils. These data suggest that HCT-3012 might be a safer alternative to nonsteroidal anti-inflammatory drugs and coxibs in rheumatic patients that take low doses of ASA.

Editorial Expression of Concern

The Journal of Pharmacology and Experimental Therapeutics is publishing an Editorial Expression of Concern regarding allegations of figure manipulation or figure duplication in several figures published in JPET. An investigation by an inquiry committee at the University of Perugia, Italy has confirmed or validated findings compatible with alleged hypotheses of electronic duplication and/or figure manipulation. The figure in question in this article is Fig. 5.

Nitric Oxide (NO)-Releasing Naproxen (HCT-3012 [(S)-6-Methoxy-α-methyl-2-naphthaleneacetic Acid 4-(Nitrooxy)butyl Ester]) Interactions with Aspirin in Gastric Mucosa of Arthritic Rats Reveal a Role for Aspirin-Triggered Lipoxin, Prostaglandins, and NO in Gastric Protection

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epi-LXA4, also termed aspirin-triggered LX or ATL (Claria and Serhan, 1995; Serhan and Oliw, 2001; Serhan et al., 2002). Similar to endogenous LXA4, ATL exerts potent anti-inflammatory actions acting as a braking signal to limit neutrophil chemotaxis and transmigration across endothelial cell layers (Serhan and Oliw, 2001) and by inducing nitric oxide (NO) synthesis (Paul-Clark et al., 2004). Local generation of ATL and NO mediate some of the anti-inflammatory activities of ASA.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used to treat pain and inflammation in OA and RA. A major limitation of their use, however, is the potential for severe gastrointestinal side effects, such as bleeding and perforation (Patrono et al., 2001). Unlike ASA that prevents arachidonic acid (AA) access to the catalytic core of COX isoenzymes by a nonreversible modification (acylation) of a serine residue near the COX-active site (Roth et al., 1975; Lecomte et al., 1994; Loll et al., 1995; Mancini et al., 1997; FitzGerald, 2003), NSAIDs deplete platelet TXA2 formation for only a limited amount of time and therefore do not reduce the risk of a first myocardial infarction (Rahme et al., 2001; Cleland, 2002). Selective COX-2 inhibitors, coxibs, a newer class of anti-inflammatory agents (FitzGerald, 2003), spare COX-1 and produce gastrointestinal ulcer complications at about half the rate of conventional NSAIDs (Bombardier et al., 2000; Silverstein et al., 2000). However, in contrast to conventional NSAIDs, coxibs not only are devoid of antiplatelet activity (FitzGerald, 2003), but their use has been linked to an increased risk of nonfatal myocardial infarction (Bombardier et al., 2000) raising the question of whether cardiovascular protection should be recommended to patients with cardiovascular risk factors that take a coxib. Although the use of low doses of ASA has been recommended (Bombardier et al., 2000), human studies suggest that coadministration of a coxib with ASA increases the risk of gastrointestinal injury (Silverstein et al., 2000; Wallace et al., 2000; Fiorucci et al., 2002, 2003b).

NO, a ubiquitous signaling molecule (Cirino, 2003), is increasingly recognized as a key mediator of gastrointestinal mucosal integrity. NO protects gastric epithelial cells against injury caused by exposure to NSAIDs in vitro (Fiorucci et al., 1999, 2001) and attenuates gastrointestinal injury in rodent models of NSAID gastropathy (Lopez-Belmonte et al., 1993). NO-releasing NSAIDs (NO-NSAIDs) are a family of anti-inflammatory drugs that inhibit COX activities while releasing NO (Fiorucci et al., 2001). HCT-3012 [(S)-6-methoxy-alpha-methyl-2-naphtalene-acetic acid 4-(nitrooxy)butyl ester] is the NO-releasing derivative of naproxen, which similar to the parent drug, inhibits formation of COX-1- and COX-2-derived prostanooids and exerts NO-mediated activities (Wallace and Cirino, 1994; Wallace et al., 1994; Cicala et al., 2000; Muscarà et al., 2000). Thus, in contrast to naproxen, HCT-3012 modulates T cell reactivity in a rodent model of RA (Cicala et al., 2000) and reduces mean arterial blood pressure in hypertensive rats (Muscarà et al., 1998). Furthermore, the administration of HCT-3012 to healthy human volunteers associates with significantly fewer endoscopic lesions than naproxen (Hawkey et al., 2003). It is unknown whether or not HCT-3012 will be proven safe for the gastric mucosa when administered in combination with ASA.

In the present study, we have investigated the anti-inflammatory activity and gastrointestinal safety of administering HCT-3012 in combination with low doses of ASA to rats rendered arthritic by the administration of Freund’s complete adjuvant (FCA).

Materials and Methods

Materials. Mycobacterium butyricum and mineral oil, Freund’s complete adjuvant, was obtained from Difco (Detroit, MI). ASA and sodium naproxen salt were obtained from Sigma-Aldrich (Milan, Italy). NO-naproxen (HCT-3012) was supplied by NicOx SA (Sophia Antipolis, France). Celecoxib was synthesized as previously described (Fiorucci et al., 2003a). Polyclonal rabbit antibody against COX-2 was from Cayman Chemical (Ann Arbor, MI), and the donkey anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase was from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). The IL-6 ELISA kit was from Pierce Endoegen (Rockford, IL).

Induction of Freund’s Adjuvant Arthritis. Arthritis (Pearson et al., 1961; Cicala et al., 2000) was induced by subcutaneous injection at the base of the tail of 100 μl of FCA (mineral oil containing 6 mg/ml of heat-killed M. butyricum) to 7- and 8-week-old male Lewis rats (Harlan Nossan, Milan, Italy). Seven days later, FCA-treated rats were randomized to receive one of the treatments listed in Table 1. All drugs were suspended in 1% carboxymethyl cellulose. Rats (6-8 per group) were administered daily by gavage from day 7 to 21 after arthritis induction. Control animals received 300 μl of 1% carboxymethyl cellulose by oral gavage. The severity of arthritis was assessed by measuring the paw volume (edema) by a hydroplethysmometer (Ugo Basile, Milan, Italy) immediately before arthritis induction (basal value) and 3, 7, 11, 14, 17, and 21 days thereafter for both hindpaws. The hindpaw swelling was expressed as the ratio between the hindpaw volume (measured in milliliters) and the animal weight (expressed in grams). To assess the severity of arthritis, the number of tail nodules was also counted through the study. Animals were sacrificed on day 21 after RA induction, and gastric mucosal injury was scored as previously described by an operator that was unaware of the treatment that the animals had received (Fiorucci et al., 1999, 2002). Briefly, the length (in millimeters) of all erosive/hemorrhagic lesions was measured with a digital caliper, and a “gastric damage score” was calculated for each stomach by summing these values. After scoring the damage, a sample of the corpus region of each stomach was excised and processed for measurement of myeloperoxidase (MPO) activity (Fiorucci et al., 1999), prostaglandin E2 (PGE2) synthesis, and ATL content (Fiorucci et al., 2002). The remainder of the stomach was fixed in formalin and processed by routine methods for light microscopy.

Gastric Eicosanoids. The corpus mucosa was isolated, weighed, and added to a tube containing 100% ethanol plus 100 μM indomethacin to prevent further synthesis of prostaglandins. Then, samples were homogenized with a polytron homogenizer and centrifuged at 12,000 rpm for 10 min at 4°C. After the supernatant of each sample had been evaporated under a nitrogen stream, the residue was resolved in an assay buffer solution and used for determination of

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Length of Treatment</th>
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<tr>
<td>1 (8)</td>
<td>No treatment (naive)</td>
<td>From day 7 to day 21</td>
</tr>
<tr>
<td>2 (12)</td>
<td>FCA alone RA</td>
<td>From day 7 to day 21</td>
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<tr>
<td>3 (8)</td>
<td>FCA, ASA 50 mg/kg/day</td>
<td>From day 7 to day 21</td>
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<tr>
<td>4 (8)</td>
<td>FCA, naproxen 10 mg/kg/day</td>
<td>From day 7 to day 21</td>
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<tr>
<td>5 (8)</td>
<td>FCA, HCT-3012 15 mg/kg/day</td>
<td>From day 7 to day 21</td>
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<tr>
<td>6 (8)</td>
<td>FCA, celecoxib 30 mg/kg/day</td>
<td>From day 7 to day 21</td>
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<td>7 (8)</td>
<td>FCA, ASA + naproxen</td>
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<td>8 (8)</td>
<td>FCA, ASA + HCT-3012</td>
<td>From day 7 to day 21</td>
</tr>
<tr>
<td>9 (8)</td>
<td>FCA, ASA + celecoxib</td>
<td>From day 7 to day 21</td>
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* Number of rats are indicated in parentheses.
The concentration of PGE2 was measured using an enzyme immunoassay (Cayman Chemical). LXA4 content was measured using commercially available ELISA kits (Neogen, Lexington, KY). The antibody used in this assay specifically recognizes 15(R)-epi-LXA4 and has been characterized previously (Chiang et al., 1998; Fiorucci et al., 2002). Plasma levels of TXB2 were quantified with commercially available ELISA kits (Neogen, Lexington, KY). The manufacturer’s protocol.

**RT-PCR Analysis.** Stomachs were removed and immediately snap-frozen in liquid nitrogen and stored at -80°C until used. Total RNA from stomach specimens were prepared using TRIzol reagent (Invitrogen, Milan, Italy) as described (Fiorucci et al., 2002). Reverse transcription of total RNA (1 μg) was performed with random hexamers and Superscript II (Invitrogen) 50 min at 42°C. The resulting single-strand cDNA was used as a template for the subsequent PCR amplification reaction. PCR experiments were carried out with 2 μl of the first-strand DNA (cDNA) in a 20-μl mixture containing: 2 μl of PCR buffer 10× (200 mM Tris-HCl pH 8.4, 50 mM KCl), 200 μM dNTP, 1.5 mM MgCl2, 1 μM specific primers pair, and 1 U of Platinum Taq DNA polymerase (Invitrogen) and RNase-free water to a 20-μl final volume using iCycler (Bio-Rad, Hercules, CA). The sequence of the sense and antisense primers for rat COX-1 (167 bp) were 5’ GCCTGCACCTACCAATGT 3’ and 5’ AGGGGCACTCAACACTACCA 3’ and rat COX-2 (214 bp) 5’ TACCGGACTCGATTGTACG 3’ and 5’ AAGTTGGTGCCGCTGCAATC 3’. PCR was carried out for 30 and 36 amplification cycles for COX-1 and COX-2, respectively, as follows: 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C. A final extension step was then performed by heating at 72°C for 5 min. The integrity of cDNA samples was confirmed using rat β-actin (198 bp) specific primers: 5’ TCACTGGCTATGGTGATG 3’ for the sense and 5’ TTAAGGTCAGGACTGATC 3’ for the antisense. Control PCR reactions also were performed on nonreverse-transcribed RNA to exclude any contamination by genomic DNA. The amplified products were detected by electrophoresis on 1.8% agarose gel stained with 0.5 μg/ml ethidium bromide. The fragment size was assessed by comparison with a 100-bp DNA ladder (Invitrogen). The gel was photographed under ultraviolet transillumination, images were then digitalized, and a semiquantitative analysis was performed using Kodak Digital Science ID image analysis software. Each assay was carried out in quadruplicate. Results were normalized and expressed as ratio of pixel density units for specific mRNA to β-actin mRNA.

Gastric COX-1 and COX-2 expression was also assessed by Western blotting analysis. For immunoblot analysis, 20 μg of protein obtained from gastric mucosal lysates were electrotransferred onto nitrocellulose filters (Bio-Rad). The membrane was then rinsed briefly with 20 mM Tris-HCl, 150 mM NaCl, pH 7.8 TBS, blocked with 5% w/v skim milk in TBST (TBS 0.05% Tween 20, pH 7.8) for 30 min at room temperature to prevent nonspecific binding and then probed overnight at 4°C with a 1:1000 dilution of monoclonal mouse anti-sheep COX-1 and COX-2 polyclonal rabbit anti-mouse (Cayman Chemical). Membranes were washed three times in TBST for 10 min each at room temperature and then incubated for 60 min with a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-hamster secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After further washes in TBST at room temperature, the blots were developed with the enhanced chemiluminescent detection reaction (ECL Western blotting kit; Amersham Biosciences UK, Ltd.) and exposed to Kodak Biomax film. Images where then digitalized and densitometric analysis of COX-1 and COX-2/β-actin immunoprecipitates were carried out using a specific software (Kodak Digital Science ID image analysis software).

**Statistical Analysis.** All data are presented as the mean ± S.E.M. with sample sizes of at least five rats per group (unless otherwise specified). Comparisons of groups of data were performed using a one-way analysis of variance followed by the Student Newman-Keuls post-hoc test. An associated probability (P value) of less than 5% was considered significant.

**Results**

**Adjuvant Arthritis.** All FCA-injected rats developed inflammation in both hindpaws (i.e., injected as well as contralateral paw). Development of arthritis as measured by paw edema (Fig. 1, a and b) was evident as early as 2 weeks after FCA administration and reached a plateau between the second and third week (n = 8–12/group; P < 0.001 versus naive). On day 21 (Fig. 2, a and b), FCA-injected rats demonstrated a mean of 16.8 ± 1.0 arthritic nodules in the tail (n = 8–12/group; P < 0.001 versus naive) and a severe wasting disease with a loss of ~25% of body weight in comparison with naive rats (n = 8–12/group; P < 0.001 versus naive).

Hindpaw swelling was significantly attenuated by administering rats with 10 mg/kg naproxen alone or in combination
with 30 mg/kg ASA \( (n = 8–12/\text{group}; P < 0.01 \text{ versus FCA}) \), as well as by 15 mg/kg HCT-3012 alone or in combination with ASA \( (n = 8–12/\text{group}; P < 0.001 \text{ versus FCA}) \). In contrast, administering rats with ASA alone failed to reverse inflammation induced by FCA \( (n = 8–12/\text{group}; P < 0.01 \text{ versus FCA}) \). None of the treatment was effective in reverting the wasting syndrome associated with arthritis development (Fig. 2a). HCT-3012 and naproxen, but not celecoxib, effectively reduced systemic inflammation as measured by counting the number of tail nodules \( (n = 8–12/\text{group}; P < 0.001 \text{ versus FCA}) \). IL-6 is a marker of systemic inflammation in RA. Consistent with this concept, a 3-fold increase in IL-6 plasma levels was documented in arthritic rats in comparison with naive animals \( (n = 8–12; P < 0.01 \text{ versus naive}) \). Administration of HCT-3012, as well as celecoxib, reduced IL-6 plasma levels (Fig. 3a), the inhibitory effect being insensitive to the addition of ASA \( (n = 6; P < 0.01 \text{ versus FCA}) \). In contrast, administering rats with naproxen, alone or in combination with ASA, had no effect on IL-6 plasma levels \( (n = 6; P > 0.05 \text{ versus FCA}) \). TxB\(_2\) generation, a measure of platelet COX-1 activity, was significantly reduced by the administration of ASA, naproxen, and HCT-3012 and by the combination of these agents (Fig. 3b). Coadministration of HCT-3012 and naproxen with ASA \( (n = 6; \text{both treatment } P < 0.01 \text{ versus arthritic rats}) \) did not cause further suppression of TxB\(_2\) generation. As expected, a significant reduction of TxB\(_2\) plasma levels was observed in arthritic rats administered ASA alone \( (n = 8; P < 0.01 \text{ versus arthritic rats}) \). Confirming the fact that platelet’s COX-1 is the main source of plasma TxB\(_2\), administering rats with celecoxib alone failed to reduce TxB\(_2\) plasma levels \( (n = 6; P > 0.05 \text{ versus arthritic rats}) \), although inhibition was observed in arthritic rats treated with the combination of celecoxib and ASA \( (n = 6; P > 0.05 \text{ versus FCA alone}) \).

**Gastric Mucosal Lesions.** Although a minor mucosal injury was detected in rats treated with FCA alone (Fig. 4a), administering FCA-injected rats with naproxen (10 mg/kg) resulted in extensive gastric damage with a mean gastric mucosal injury score of 22.1 ± 4.6 mm \( (n = 8–12; P < 0.01 \text{ versus arthritic rats}) \). Administration of 15 mg/kg HCT-3012, i.e., equimolar with the dose of naproxen, caused significantly less injury than naproxen \( (6.8 ± 0.9 \text{ mm } (n = 8–12, P < 0.01 \text{ versus naproxen}) \). Although treating rats with ASA alone caused mild gastric injury \( (11.0 ± 1.9 \text{ mm } (n = 8–12; P < 0.05 \text{ versus arthritic rats}) \), the combined administration of naproxen with ASA resulted in extensive mucosal injury that was significantly higher than damage caused by ASA alone, indicating that the two agents synergize to produce mucosal injury.
In contrast, coadministration of HCT-3012 in combination with ASA did not exacerbate gastric mucosal injury caused by ASA alone resulting in a gastric mucosal injury score of 11.2 ± 2.1 mm (n = 8; P < 0.05 versus ASA alone). Celecoxib caused similar damage than HCT-3012, but the gastric injury caused by this agent was significantly enhanced by the coadministration of ASA (n = 8; P < 0.01 versus ASA alone).

As illustrated in Fig. 4b, administration of naproxen alone or in combination with ASA significantly increased gastric MPO activity, a measure of neutrophils margination within the gastric microcirculation (P < 0.01 versus FCA-injected rats). In contrast, no significant increase of gastric MPO activity was detected in animals treated with HCT-3012 alone (n = 8; P < 0.001 versus FCA alone). Cotreating arthritic rats with ASA in combination with HCT-3012 increased MPO activity significantly in comparison with HCT-3012 alone, although this figure was significantly lower than that observed in rats taking ASA in combination with naproxen (n = 8; P < 0.01 versus ASA plus naproxen). Although celecoxib alone did not increase MPO activity, its coadministration, in combination with ASA, enhanced neutrophil accumulation into the gastric mucosa (n = 8–12; P < 0.01 versus arthritic rats).

**Gastric COX-1 and COX-2 Expression.** As illustrated in Fig. 5a–c, although the expression of gastric COX-1, mRNA, and protein did not differ among groups, COX-2 expression, mRNA, and protein was significantly enhanced in the stomach of arthritic rats on day 21 following the FCA injection (Fig. 5, a, b, and d). COX-2 expression, mRNA, and protein
were further enhanced by treating arthritic rats with ASA, naproxen, and the combination of the two. In contrast, no significant induction of COX-2 was detected in rats treated with HCT-3012 alone or in combination with ASA. Finally, whereas celecoxib alone did not up-regulate COX-2 expression, this effect was evident in rats coadministered celecoxib in combination with ASA.

**Gastric Mucosal Eicosanoids.** Gastric PGE$_2$ generation was significantly enhanced in arthritic rats in comparison with naive animals ($n = 8–12; P < 0.001$ versus arthritic rats). Despite the fact that HCT-3012 was better tolerated than naproxen, it inhibited PGE$_2$ generation to the same extent than its parent drug ($n = 6; P < 0.001$ versus FCA-injected rats). A significant inhibition of gastric PGE$_2$ formation was documented in rats treated with celecoxib ($n = 6; P < 0.001$ versus arthritic rats), thereby supporting the view that in arthritic rats, gastric COX-2 contributes to a generation of mucosal prostanooids. Cotreating rats with ASA in combination with HCT-3012 or naproxen did not induce a further reduction of PGE$_2$ concentration ($n = 6; P < 0.001$ versus FCA-injected rats). Administering ASA to arthritic rats resulted in a significant increase in gastric ATL content, an effect that was almost completely abrogated by cotreating rats with HCT-3012, naproxen, and celecoxib ($n = 6; P < 0.001$ versus ASA alone) (Fig. 6).

**Discussion.**

Administration of low-dose ASA to inhibit platelet TXA$_2$ biosynthesis is of proven efficacy for prevention of cardiac ischemic events (Patrono, 1994). Although low doses of ASA inhibits COX-1 in platelets, they largely spare systemic COX-2 activity, raising the question of whether they might be used in association with selective and nonselective COX-2 inhibitors to treat pain and inflammation in patients with RA and OA with cardiovascular risk factors (Patrono, 1994; FitzGerald, 2003). However, results from a large clinical trial indicate that the use of ASA might increase the risk of gastrointestinal bleeding in patients with RA and OA taking celecoxib (Silverstein et al., 2000).

In the present study, we have shown that administration of selective and nonselective COX-2 inhibitors to arthritic rats treated with a dose of ASA that cause 70% inhibition of TXA$_2$ formation, exacerbates gastric damage induced by ASA by a mechanism that involves the inhibition of formation of mediator(s) generated by the acetylated form of COX-2 (Claria and Serhan, 1995; Serhan and Oliw, 2001). ASA prevents AA access to the catalytic core of COX isoenzymes by covalently modifying a serine residue near the COX-active site of the enzyme (Lecomte et al., 1994). However, although acetylation of serine residue 530 of human COX-1 abolishes the enzyme’s capability to oxidize AA, the acetylation of the corresponding serine residue in COX-2 (Ser 516) modifies the enzyme such that it performs an incomplete reaction in which AA is converted to 15-HETE carrying its C15 alcohol in the $R$ configuration [15($R$)-HETE] (Serhan and Oliw, 2001). 15($R$)-HETE derived from ASA-acetylated COX-2 can be converted by lipoxygenase isoforms into 15($R$)-epi-lipoxin A$_4$ (15-epi-LXA$_4$) also known as aspirin-triggered lipoxin or ATL (Claria and Serhan, 1995; Serhan and Oliw, 2001). ATL is a potent counter-regulatory agent and mediates some of the anti-inflammatory activities of ASA at the leukocytes/endothelial cell interface (Claria and Serhan, 1995; Fiore and Serhan, 1995; Takano et al., 1997, 1998; Filep et al., 1999; Serhan and Oliw, 2001; Fiorucci et al., 2003a). Indeed, ATL (and its metabolically stable analogs) inhibit granulocytes recruitment at the site of inflammation by dampening the release of proinflammatory chemokines/ cytokines, as well as by modulating the expression of adhesion molecules on neutrophils and endothelial cells (Jozsef et al., 2002). A growing body of evidence indicate that, similar to other members of the resolvin family (Serhan et al., 2002), ATL promotes resolution of inflammation and has the capability to inhibit the activity of transcription factors such as the adaptor protein-1 and nuclear factor-$\kappa$B (Jozsef et al., 2002). We have previously shown by favoring neutrophils disengagement from the gastric microcirculation, ATL is also involved in the development of gastric adaptation to ASA (Fiorucci et al., 2002). The demonstration that ATL induces NO formation (Paul-Clark et al., 2004) further support the anti-inflammatory and gastrosparing role of ATL in animal models of NSAID gastropathy. The present results add to this concept by demonstrating a critical role of ATL in gastric adaptation to low doses of ASA in a rodent model of arthritis. In this model, arthritis development associates with induction of gastric COX-2, likely as a result of systemic inflammation and high plasma levels of proinflammatory cytokines such as IL-6 (Davies et al., 1997; Kato et al., 1999, 2001). Thus,
although COX-2 was barely detectable in the mucosa of intact rats, its expression, mRNA, and protein was markedly enhanced in arthritic rats, an event that correlates with a significant increase in gastric PGE$_2$ synthesis. Gastric COX-2 expression was even further increased in arthritic rats exposed to ASA (Davies et al., 1997) alone or in combination with naproxen and celecoxib, but not in rats treated with HCT-3012 alone or in combination with ASA or celecoxib alone.

Although all NSAIDs used in this study, being selective or not for COX-2, reduced gastric PGE$_2$ synthesis, celecoxib was significantly better tolerated than naproxen, suggesting that COX-1-derived prostanoids might compensate for selective COX-2 inhibition in this experimental model. However, when COX-1 activity was suppressed by feeding arthritic rats a low dose of ASA, we found that administration of celecoxib, similarly to naproxen, exacerbated mucosal injury. These data are consistent with previous studies indicating that simultaneous inhibition of COX-1 and COX-2 is required to cause gastric damage in rodents (Wallace et al., 2000). Because the combination of ASA and celecoxib causes a simultaneous inhibition of PGE$_2$ and ATL synthesis, our results strongly support the notion that inhibition of acetylated COX-2, in the context of COX-1 suppression, is the main mechanism involved in exacerbation of gastric injury caused by celecoxib and, by extension, by nonselective COX-2 inhibitors. Supporting this view, we have previously shown that LXA$_4$ analog rescues rats from injury caused by ASA, whereas administering rats with Boc-1, a LXA$_4$ receptor antagonist, exacerbates the injury (Fiorucci et al., 2002).

An interesting observation made in this study was the demonstration that administration of a COX inhibitor to arthritic rats potentiates the chemoattractant effect of ASA on neutrophils (McCafferty et al., 1995). Indeed, we observed a ∼4-fold increase of MPO activity in the gastric mucosa of arthritic rats treated with ASA plus a COX inhibitor, but not HCT-3012, in comparison with rats treated with ASA alone. This synergistic effect on neutrophil attraction is most likely the consequence of inhibition of ATL formation. Previous studies have demonstrated that ATL counteracts vascular leakage and neutrophil trafficking as well as neutrophil recruitment induced by proinflammatory stimuli such as leukotriene B$_4$, tumor necrosis factor-α, and IL-6 (Claria and Serhan, 1995; Fiore and Serhan, 1995; Takano et al., 1997, 1998; Clish et al., 1999; Filep et al., 1999; Serhan and Oliw, 2001; Fiorucci et al., 2003a).

HCT-3012 is a prototype of a new class of anti-inflammatory agents coupled with NO. NO-NSAIDs retain the classic therapeutic profile of native compounds such as the ability to inhibit inflammatory response, nociception, and fever, but spare the gastrointestinal tract (Wallace and Cirino, 1994; Davies et al., 1997; Wallace et al., 1997). In the present study, we have shown that similar to naproxen, HCT-3012 administered in a therapeutic manner reduces joint edema formation and systemic inflammation in arthritic rats. Furthermore, confirming previous animal data (Cicala et al., 2000), our data demonstrated that in contrast to naproxen, HCT-3012 was effective in reducing circulating levels of IL-6, a well recognized marker of systemic inflammation in this model. Animal studies have shown that NO-NSAIDs spare the stomach at doses that completely inhibit gastric mucosal COX-1 activity (Wallace et al., 1994), suggesting that mechanisms other than gastric mucosal prostaglandin preservation are involved in gastrointestinal protection afforded by these drugs. Topical application of NSAIDs decrease gastric mucosal blood flow, an event that leads to neutrophil recruitment in the gastric microcirculation and plays a mechanistic role in the pathogenesis of NSAID gastropathy. NO-releasing NSAIDs, including HCT-3012, maintain gastric mucosal blood flow (Wallace et al., 1994) and inhibit neutrophil function and down-regulates the expression of adhesion molecules required for leukocyte adherence to the endothelium, an important step involved in the process of targeting neutrophils to the gastric microcirculation (De Caterina et al., 1995; Khan et al., 1996). Here, we have provided evidence that although HCT-3012 exerts a potent anti-inflammatory activity and inhibits gastric PGE$_2$ synthesis, it did not injure the gastric mucosa of arthritic rats. Furthermore, in contrast to celecoxib and naproxen, HCT-3012 did not exacerbate gastric mucosal injury caused by cotreatment of arthritic rats with low doses of ASA. In contrast with celecoxib and naproxen, HCT-3012 did not increase neutrophil margination into the stomach when given alone nor did it potentiate gastric neutrophil accumulation when administered in combination with ASA, suggesting that this compound exerts COX-independent, NO-mediated effects.

Consistent with the fact that HCT-3012 inhibits COX-1 and COX-2, it also suppresses ATL formation when coadministered in combination with ASA to arthritic rats. Although these data indicate that HCT-3012 inhibits the acetylated and nonacetylated form of COX-2, it appears that NO released by its NO-donating moiety has the potential to compensate for ATL and PGE$_2$ deficiency in this experimental setting.

In summary, by demonstrating that ASA increases ATL formation in arthritic rats, we have provided evidence that acetylated COX-2 might play a role in an animal model relevant to a human disease. We have also shown that simultaneous administration of a low dose of ASA in combination with celecoxib increases the tendency of ASA to cause gastric injury in the rodent model of RA. Finally, our results support the notion that NO released from the NO-donating moiety of HCT-3012 attenuates gastric injury even in the absence of PGE$_2$ and ATL.

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