Aspirin, but not NO-releasing aspirin (NCX-4016), interacts with selective COX-2 inhibitors to aggravate gastric damage and inflammation

John L. Wallace,1 Stella R. Zamuner,1 Webb McKnight,1 Michael Dicay,1 Andrea Mencarelli,2 Piero del Soldato,3 and Stefano Fiorucci2

1Mucosal Inflammation Research Group, University of Calgary, Calgary, Alberta, Canada T2N 4N1; 2Department of Gastroenterology and Hepatology, University of Perugia, 06122 Perugia, Italy; and 3NicOx, 06906 Sophia Antipolis, France

Submitted 15 July 2003; accepted in final form 7 August 2003

Wallace, John L., Stella R. Zamuner, Webb McKnight, Michael Dicay, Andrea Mencarelli, Piero del Soldato, and Stefano Fiorucci. Aspirin, but not NO-releasing aspirin (NCX-4016), interacts with selective COX-2 inhibitors to aggravate gastric damage and inflammation. Am J Physiol Gastrointest Liver Physiol 286: G76–G81, 2004; 10.1152/ajpgi.00295.2003.—Acetylation of cyclooxygenase (COX)-2 by aspirin can trigger the formation of 15(R)-epi-lipoxin A4, or aspirin-triggered lipoxin (ATL). ATL exerts protective effects in the stomach. Selective COX-2 inhibitors block ATL synthesis and exacerbate aspirin-induced gastric damage. Nitric oxide-releasing aspirins, including NCX-4016, have antiplatelet effects similar to aspirin but do not cause gastric damage. In the present study, we examined whether or not NCX-4016 triggers ATL synthesis and/or upregulates gastric COX-2 expression and the effects of coadministration of NCX-4016 with a selective COX-2 inhibitor on gastric mucosal injury and inflammation. Rats were given aspirin or NCX-4016 orally and either vehicle or a selective COX-2 inhibitor (celecoxib) intraperitoneally. Gastric damage was blindly scored, and granulocyte infiltration into gastric tissue was monitored through measurement of myeloperoxidase activity. Gastric PG and ATL synthesis was measured as was COX-2 expression. Whereas celecoxib inhibited gastric ATL synthesis and increased the severity of aspirin-induced gastric damage and inflammation, coadministration of celecoxib and NCX-4016 did not result in damage or inflammation. NCX-4016 did not upregulate gastric COX-2 expression nor did it trigger ATL synthesis (in contrast to aspirin). Daily administration of aspirin for 5 days resulted in significantly less gastric damage than that seen with a single dose, as well as augmented ATL synthesis. Celecoxib reversed this effect. In contrast, repeated administration of NCX-4016 failed to cause gastric damage, whether given alone or with celecoxib. These studies support the notion that NCX-4016 may be an attractive alternative to aspirin for indications such as cardioprotection, including in individuals also taking selective COX-2 inhibitors.

cyclooxygenase-2; nitric oxide; lipoxin; ulcer; neutrophil
observed with aspirin? We also examined the possibility that neutrophil infiltration of the gastric mucosa after aspirin administration represents an important source of COX-2.

METHODS AND MATERIALS

Animals. Male Wistar rats weighing 175–200 g were obtained from Charles River Breeding Farms (Montreal, Canada or Monza, Italy). The rats were fed standard laboratory chow and tap water. All experimental procedures described below were approved by our institutional animal research committees and were performed in accordance with nationally approved guidelines for the treatment of laboratory animals. In all experiments described below the sample size in each group was at least 5.

Effects of aspirin and NCX-4016. Rats were deprived of food for 18–20 h with free access to drinking water and were treated orally with aspirin (10, 30, or 100 mg/kg), equimolar doses of NCX-4016, or vehicle (1% carboxymethylcellulose). Three hours later, the rats were euthanized for blind assessment of gastric damage (28). The lengths (in mm) of all hemorrhagic lesions were measured with digital calipers, and the “gastric damage score” was calculated for each stomach by summing these values. After the damage was scored, a sample of the corpus region of each stomach was fixed in neutral buffered formalin for subsequent histological assessment. Another tissue sample was excised for measurement of MPO activity (15) by using a commercially available spectrophotometric assay. MPO is an enzyme found primarily in the azurophilic granules of neutrophils and therefore has been used extensively as a biochemical marker of granulocyte infiltration into various tissues including the gastrointestinal tract. Another tissue sample from each stomach was immediately frozen in liquid nitrogen and then stored at −80°C for subsequent measurement of tissue levels of PGE2 (23) and 15(R)-epi-LXA4 (9).

Effects of COX-2 inhibition. As in the studies above, rats were treated orally with aspirin (10, 30, or 100 mg/kg), NCX-4016 (equimolar doses), or vehicle and were killed 3 h later for assessment of gastric damage. However, these rats were also treated intraperitoneally with celecoxib (10 mg/kg) at the same time as the oral dosing with aspirin. We also examined the possibility that effects of aspirin on sodium azide and dryness under a stream of nitrogen and then reconstituted in 1 ml of 0.1 M phosphate buffer (pH 7.4) containing 0.8% sodium azide and 0.1% gelatin. Concentration of PGE2 in these samples was then measured by using a highly specific, commercially available ELISA (21). The mixture of 3.0 ml of extraction solvent (isopropanol/ethyl acetate/0.1 M phosphate buffer (pH 7.4) containing 0.8% sodium azide and 0.1% gelatin) was aspirated and divided into two tubes. The sample was then dried under a stream of nitrogen and then reconstituted in 1 ml of 0.1 M phosphate buffer (pH 7.4) containing 0.8% sodium azide and 0.1% gelatin. Concentration of PGE2 in these samples was then measured by using a commercially available spectrophotometric assay. MPO is an enzyme found primarily in the azurophilic granules of neutrophils and therefore has been used extensively as a biochemical marker of granulocyte infiltration into various tissues including the gastrointestinal tract. Another tissue sample from each stomach was immediately frozen in liquid nitrogen and then stored at −80°C for subsequent measurement of tissue levels of PGE2 (23) and 15(R)-epi-LXA4 (9).

Measurement of COX-2 and 15(R)-epi-LXA4. The frozen tissue samples (100–150 mg) of the gastric corpus were homogenized in a mixture of 3.0 ml of extraction solvent (isopropanol/ethyl acetate/0.1 N HCl, 3.3:1) and 3.0 ml of distilled water. Homogenates were centrifuged at 1,500 g for 10 min at 4°C. The organic phase was aspirated and divided into two tubes. The first was evaporated to dryness under a stream of nitrogen and then reconstituted in 1 ml of 0.1 M phosphate buffer (pH 7.4) containing 0.8% sodium azide and 0.1% gelatin. Concentration of PGE2 in these samples was then measured by ELISA by using a commercially available kit. The other half of each sample was diluted 1:5 with water and acidified to pH 3.5 with 1 N HCl. Samples were applied to a preconditioned C18 Sep-Pak column (Waters, Mississauga, ON, Canada), and after being washed with 1 ml of water followed by 1 ml of petroleum ether, lipoxins were eluted with 2 ml of methyl formate. The sample was then dried under a stream of nitrogen and reconstituted in assay buffer.

Concentrations of 15(R)-epi-LXA4 in the samples were measured by using a highly specific, commercially available ELISA (21). The antibody used in this ELISA is specific for 15(R)-epi-LXA4, with cross-reactivity with 15(S)-HETE and 15(R)-HETE being 0.13 and 1.25%, respectively. With the use of reverse-phase high-performance liquid chromatography, we have confirmed that the rat stomach produces 15(R)-epi-LXA4 after aspirin administration in a COX-2-dependent manner (9).

COX-2 expression. Gastric COX-2 expression was examined by Western blot analysis. Samples of gastric tissue were homogenized in lysis buffer (0.1% Triton X-100, 50 μM pepstatin-A, 0.2 mM leupeptin, 1 μg/ml aprotinin, 10 mg/ml phenylmethyl sulfonyl fluoride, 50 mM Tris, and 10 mM EDTA). Samples were then centrifuged and protein concentration of the supernatant was determined by colorimetric assay (Bio-Rad, Hercules, CA). Protein (50 μg) was separated on a 10% polyacrylamide gel and then transferred to a nitrocellulose membrane (1). The membrane was incubated for 1 h with blocking buffer (20 mM Tris, 100 mM NaCl, 0.5% Tween 20, and 5% nonfat dried milk) and then probed overnight with a polyclonal rabbit antibody against COX-2 (1:500; Cayman Chemical, Ann Arbor, MI). The membrane was then incubated with a donkey anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Amersham, Little Chalfont, UK) for 1 h at room temperature. A chemiluminescence reagent (Amersham) was added to visualize the labeling according to the manufacturer’s instructions. Densitometry was done by using a calibrated imaging densitometer (model GS-710; Bio-Rad) and analyzed with Quantity One software (Bio-Rad).

RT-PCR analysis. Total RNA was prepared by using RNeasy Fibrous Tissue Kit (Qiagen SpA, Milan, Italy) according to the manufacturer’s instructions. Traces of genomic DNA that may copurify with RNA were removed by a DNase treatment on the same spin column provided with the kit. Total RNA was reverse-transcribed by using random hexamers as described previously (8). One-tenth of each cDNA sample was amplified by PCR with COX-1- and COX-2-specific primers. Each sample contained the sense and antisense primers (1 μM), 200 μM dNTPs, 50 mM KCl, 20 mM Tris-HCl (pH 8.6), 1.5 mM MgCl2, and 1.25 units platinum Taq polymerase (In Vitrogen Life Technologies, Milan, Italy). Thermal cycling was performed for 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C. After 24, 26, 28, 30, and 32 cycles, 30-s pauses were programmed to obtain 5-μl samples. The sense and antisense primers were 5’-GCCTCGACCACTTACAAATGT-3’ and 5’-AGGTGGCATTCAAAACTCTCC-3’ for COX-1 (167 bp), and 5’TACCCGGACTTCTCTTGGAG-3’ and 5’-AGGTGGTGCCGGCTGCAATC-3’ for COX-2 (214 bp). For rat β-actin, the primer pairs used were 5’-TGTTGATGGTGGAAATGG-3’ and 5’T-ATTGGTGCGCCAGATATTCC-3’. PCR reactions also were performed on nonreverse-transcribed RNA to exclude any contamination by genomic DNA. Amplified DNA was analyzed on a 1.8% agarose gel. Fragment size was assessed by comparison to 100 bp DNA marker (Invitrogen). Gel was photographed under ultraviolet transillumination with a Kodak Digital Science ID Image Analysis Software. The images were then digitized and a semiquantitative analysis was performed by using the same software.

Statistical analysis. All data are expressed as mean ± SE. Groups of data were compared by using a one-way ANOVA followed by the Student-Newman-Keuls test. An associated probability (P value) of <5% was considered significant.

Materials. Aspirin was obtained from Sigma (St. Louis, MO). Celecoxib and NCX-4016 were provided by Nicox (Sophia Antipolis, France). The ELISA kit for PGE2 was obtained from Cayman Chemical, whereas that for ATL [15(R)-epi-LXA4] was obtained from Neogen (Lexington, KY). The kits for measurement of MPO activity were obtained from CytoStore (Calgary, AB, Canada). All other materials were obtained from Fisher Scientific (Edmonton, AB, Canada).

RESULTS

Aspirin caused the formation of hemorrhagic lesions in the corpus region of the stomach, as described previously (17, 25), which increased in severity in a dose-dependent manner (Fig. 1). In contrast, equimolar doses of NCX-4016 did not produce any detectable gastric damage. The absence of damage after administration of NCX-4016 was confirmed by histological evaluation. Lack of damage with NCX-4016 occurred despite inhibition of gastric PG synthesis to the same extent as equimo-
lar doses of aspirin (Table 1). Administration of celecoxib at a dose of 10 mg/kg did not cause any detectable gastric damage (mean damage score of 0 ± 0; n = 5). Coadministration of celecoxib and aspirin caused a significantly greater degree of gastric damage than was seen with aspirin alone (Fig. 1). In contrast, the combination of celecoxib and NCX-4016 did not cause detectable gastric damage. Celecoxib did not significantly affect gastric PGE₂ levels, whether given alone or in combination with aspirin or NCX-4016 (Table 1).

In addition to increasing the severity of gastric damage caused by aspirin, coadministration of celecoxib also significantly increased the extent of granulocyte infiltration into the stomach as measured by MPO activity (Fig. 2). In contrast, NCX-4016 did not significantly affect gastric MPO activity whether given alone or in combination with aspirin or NCX-4016 (Table 1).

Aspirin administration resulted in a marked increase in COX-2 mRNA and protein expression in the stomach (Fig. 3). In contrast, administration of NCX-4016 did not result in any change in COX-2 expression. Given the observation that gastric MPO activity increased markedly after aspirin administration and previous reports that COX-2 is expressed in infiltrating neutrophils (20, 21), we examined the possibility that infiltrating neutrophils were the source of the increase in COX-2 expression in the stomach after aspirin administration. Groups of 5 rats each were treated intraperitoneally with antineutrophil serum (Accurate Chemical, Westbury, NY) or normal rabbit serum 24 and 2 h before oral administration of aspirin (100 mg/kg) or vehicle. We have previously demonstrated that this dose of the antineutrophil serum reduces circulating neutrophil numbers by >95%, whereas not significantly affecting blood levels of eosinophils, macrophages,

Table 1. Effects of aspirin, NCX-4016 and celecoxib on gastric prostaglandin synthesis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PGE₂, pg/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>234 ± 15</td>
</tr>
<tr>
<td>Celecoxib (10 mg/kg)</td>
<td>246 ± 10</td>
</tr>
<tr>
<td>Aspirin (10 mg/kg) + vehicle</td>
<td>64 ± 14*</td>
</tr>
<tr>
<td>Aspirin (10 mg/kg) + celecoxib</td>
<td>71 ± 8*</td>
</tr>
<tr>
<td>Aspirin (30 mg/kg) + vehicle</td>
<td>25 ± 5*</td>
</tr>
<tr>
<td>Aspirin (30 mg/kg) + celecoxib</td>
<td>31 ± 8*</td>
</tr>
<tr>
<td>Aspirin (100 mg/kg) + vehicle</td>
<td>17 ± 4*</td>
</tr>
<tr>
<td>Aspirin (100 mg/kg) + celecoxib</td>
<td>15 ± 6*</td>
</tr>
<tr>
<td>NCX-4016 (18 mg/kg) + vehicle</td>
<td>83 ± 11*</td>
</tr>
<tr>
<td>NCX-4016 (18 mg/kg) + celecoxib</td>
<td>94 ± 8*</td>
</tr>
<tr>
<td>NCX-4016 (54 mg/kg) + vehicle</td>
<td>44 ± 7*</td>
</tr>
<tr>
<td>NCX-4016 (54 mg/kg) + celecoxib</td>
<td>51 ± 9*</td>
</tr>
<tr>
<td>NCX-4016 (180 mg/kg) + vehicle</td>
<td>24 ± 7*</td>
</tr>
<tr>
<td>NCX-4016 (180 mg/kg) + celecoxib</td>
<td>27 ± 9*</td>
</tr>
</tbody>
</table>

Values are means ± SE of 5–6 rats per group. There were no significant differences between the levels of gastric PGE₂ in rats treated with aspirin vs. rats treated with an equimolar dose of NCX-4016. Cotreatment with celecoxib (10 mg/kg) did not significantly affect the degree of suppression of PGE₂ synthesis as compared to that observed with aspirin or NCX-4016 alone. *P < 0.001 vs. the vehicle-treated group.
monocytes, or lymphocytes (29). As shown in Fig. 4, admin-
istration of aspirin to rats pretreated with antineutrophil serum
still resulted in a marked increase in gastric COX-2 expression.
This occurred despite the fact that the neutropenic rats exhib-
ited significantly less (P < 0.01) aspirin-induced gastric dam-
age (damage score of 0.6 ± 0.4) than the control rats (32.2 ± 5.2) and despite the neutropenic rats exhibiting no increase in
gastric MPO activity after aspirin administration (7.2 ± 1.4
U/mg vs. 6.1 ± 1.1 in control rats treated with vehicle and
16.1 ± 2.8 U/mg in control rats treated with aspirin).

Aspirin administration resulted in a significant increase in
ATL formation by the stomach (Fig. 5A). Celecoxib alone did
not affect ATL formation, but when given together with
aspirin, the level of ATL formation was not significantly
different from that in rats not treated with aspirin. Treatment
with NCX-4016 did not significantly affect ATL formation by
the stomach either when given alone or when given in combi-
nation with celecoxib (Fig. 5A).

Administration of aspirin each day for 5 days resulted in
hemorrhagic damage in the stomach that was significantly less
extensive than that seen after only a single dose of aspirin (Fig.
6). Suppression of gastric PG levels after 5 days of aspirin
administration was comparable to that after a single dose (20 ±
6 pg/mg after 5 days of aspirin, vs. 222 ± 19 pg/mg after 5
days of vehicle administration; see Table 1 for single dose
data). Administration of celecoxib on the final day of aspirin
administration significantly increased the extent of gastric
damage to the same level as was observed after a single dose
of aspirin. There was no further suppression of gastric PG
synthesis in rats given celecoxib and aspirin (23 ± 6 pg/mg)
compared with rats given aspirin alone for 5 days. As was the
case with single dosing of NCX-4016, daily administration of
this compound for 5 days failed to elicit any detectable gastric
damage but significantly suppressed gastric PG synthesis.
neutrophils in gastric COX-2 expression was attributable to the increase in related to the ability of this drug to release NO, because NO COX-2 expression after administration of NCX-4016 may be increased in COX-2 expression. Absence of an upregulation of gastric PG synthesis by NCX-4016, we anticipated an increase in ATL formation compared with vehicle-treated controls. Celecoxib abolished the increase in ATL formation in rats given NCX-4016 daily for 5 days (Fig. 5B).

**DISCUSSION**

Exacerbation of aspirin-induced gastric damage by a selective COX-2 inhibitor has been demonstrated in rats (9) and in healthy human volunteers (10). In the present study, we confirmed that the selective COX-2 inhibitor celecoxib markedly increased the severity of aspirin-induced gastric damage in the rat. Furthermore, we have demonstrated that an NO-releasing derivative of aspirin, NCX-4016, did not cause gastric damage whether given alone or in combination with celecoxib. Absence of damage with NCX-4016 was not due to a lack of effect on gastric PG synthesis, because at equimolar doses it suppressed gastric PG levels to the same extent as aspirin.

COX-2 suppression also resulted in a significant increase in aspirin-induced granulocyte infiltration into the stomach. Again, NCX-4016 did not increase granulocyte numbers in the stomach when given alone or with celecoxib. The absence of an increase in neutrophil infiltration into the stomach after administration of NCX-4016, despite significant suppression of gastric PG levels, may be related to the previously observed inhibitory effect of this compound on leukocyte adherence to the vascular endothelium (29). NCX-4016 significantly suppressed formyl peptide-induced leukocyte adherence in mesenteric venules (29).

NCX-4016 failed to cause the elevation in COX-2 expression observed after administration of aspirin. Several years ago, we (4) reported that aspirin could rapidly induce an upregulation of COX-2 in the stomach, and at that time we postulated that this was a compensatory response to suppressed mucosal levels of PGs, because the increase could be suppressed by administration of PGE2. Given the suppression of gastric PG synthesis by NCX-4016, we anticipated an increase in COX-2 expression. Absence of an upregulation of gastric COX-2 expression after administration of NCX-4016 may be related to the ability of this drug to release NO, because NO has been shown to suppress endotoxin-induced COX-2 expression (7). We also examined the possibility that the increase in gastric COX-2 expression was attributable to the increase in neutrophil infiltration into the mucosa, because COX-2 has previously been found to be highly expressed in infiltrating neutrophils (16, 21). However, administration of aspirin to neutropenic rats was still associated with elevated COX-2 expression. Because the neutropenic rats exhibited negligible gastric damage after aspirin administration, we can conclude that the increase in COX-2 expression induced by aspirin does not occur purely as a response to injury.

One of the main questions addressed in this study was whether or not NCX-4016, like aspirin, would trigger ATL synthesis? Despite the fact that a single administration of NCX-4016 suppressed gastric PG synthesis as effectively as aspirin it did not elicit significant formation of ATL. The vast majority of PG synthesis in the normal rat stomach occurs via COX-1 (28). It is possible that NCX-4016 inhibited COX-2 less effectively than aspirin or perhaps through a mechanism not involving acetylation of the enzyme, but this is not yet clear. ATL has recently been suggested to contribute to the reduced gastric injury that occurs after repeated administration of aspirin (6). In the present study, daily administration of aspirin for 5 days caused significantly less gastric damage than was seen after a single administration. Repeated administration of aspirin also caused more ATL formation than with a single administration, and the gastric “adaptation” was reversed when celecoxib was given at the same time as the final administration of aspirin, consistent with previous observations (6). Although a single administration of NCX-4016 did not cause ATL formation, daily administration of NCX-4016 for 5 days did result in significant generation of ATL. However, the ATL produced after repeated administration of NCX-4016 did not appear to make a significant contribution to gastric adaptation, because coadministration of celecoxib did not increase gastric damage in rats given NCX-4016 daily for 5 days.

In contrast, the reduced gastric damage observed after 5 days of aspirin administration, compared with the damage induced by a single dose, may have been, in part, related to enhanced ATL formation. ATL formation was elevated in the 5-day group vs. the single-dose group. Administration of celecoxib at the time of the final dose of aspirin increased gastric damage to the level seen in rats given only a single dose of aspirin. However, we cannot rule out other mechanisms as contributors to the adaptation of the stomach to repeated aspirin administration.

In summary, NCX-4016 does not cause significant gastric damage or inflammation even when given in combination with a selective COX-2 inhibitor. The lack of damage with this compound does not appear to be related to the triggering of lipoxin synthesis in the stomach, as occurs after aspirin administration. Gastric safety of NCX-4016 and its failure to cause significant damage when given in combination with a selective COX-2 inhibitor have been confirmed in a recent clinical study (11). Because NCX-4016 suppresses platelet function and thrombus formation as effectively as aspirin (10, 30), it represents an attractive alternative to aspirin for cardioprotective applications (24) including in patients taking selective COX-2 inhibitors.

**DISCLOSURES**

J. L. Wallace is an Alberta Heritage Foundation for Medical Research Scientist and is supported by research grants from the Canadian Institutes for Health Research (CIHR). Dr. Zamuner is supported by a Fellowship cosponsored by Janssen Pharmaceutica, the Canadian Association for Gastroenterology and the CIHR.

**REFERENCES**

2. Chiang N, Takano T, Clish C, Petasis N, Tai HH, and Serhan C. Aspirin triggered 15-epi-lipoxin A4 (ATL) generation by human leuko-

**AJP-Gastrointest Liver Physiol • VOL 286 • JANUARY 2004 • www.ajpgi.org**