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

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Wallace, John L., Stella R. Zamuner, Webb McKnight, Michael Dicy, 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 (cecloxib) 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 cecloxic inhibited gastric ATL synthesis and increased the severity of aspirin-induced gastric damage and inflammation, coadministration of cecloxic 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. Ceczolixib reversed this effect. In contrast, repeated administration of NCX-4016 failed to cause gastric damage, whether given alone or with cecloxic. 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

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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 15R-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 was performed. In the rat, this dose of celecoxib significantly inhibits COX-2 but not COX-1 (28).

Measurement of PGE2 and 15R-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 N HCl, 3:3:1) and 3.0 ml of distilled water. Homogenates were centrifuged at 1,500 rpm 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 determined by using a spectrophotometric assay. COX-2 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 15R-epi-LXA4 (9).

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 equimolar doses of aspirin.
Fig. 1. Gastric damage induced in the rat stomach by oral administration of aspirin (ASA) or NCX-4016, alone or in combination with celecoxib (10 mg/kg). Doses of NCX-4016 are equimolar to the corresponding doses of ASA. Damage was blindly assessed 3 h after drug administration. Celecoxib alone did not cause detectable gastric damage. Each bar represents the mean ± SE of 5–6 rats. *P < 0.05, **P < 0.01 vs. the corresponding group treated with ASA alone.

Fig. 2. Gastric myeloperoxidase activity measured as an index of granulocyte infiltration in rats treated with ASA or NCX-4016, alone or in combination with celecoxib (10 mg/kg). Tissue samples were taken 3 h after drug administration. Each bar represents the mean ± SE of 5–6 rats. *P < 0.05 vs. the corresponding group treated with vehicle rather than celecoxib.

Fig. 3. Expression of COX-2 mRNA (A; RT-PCR) and protein (B; by Western blotting; n = 5 per group) in the rat stomach 3 h after oral administration of vehicle (control), ASA (30 mg/kg), or NCX-4016 (54 mg/kg). Only ASA caused a significant increase in cyclooxygenase (COX)-2 expression (*P < 0.05 vs. the vehicle-treated group). For the RT-PCR gel, 3 lanes are shown for each treatment (sample from 1 rat per lane). The molecular weight markers are shown in the far left lane.

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) + vehicle</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.

Fig. 1. Gastric damage induced in the rat stomach by oral administration of aspirin (ASA) or NCX-4016, alone or in combination with celecoxib (10 mg/kg). Doses of NCX-4016 are equimolar to the corresponding doses of ASA. Damage was blindly assessed 3 h after drug administration. Celecoxib alone did not cause detectable gastric damage. Each bar represents the mean ± SE of 5–6 rats. *P < 0.05, **P < 0.01 vs. the corresponding group treated with ASA alone.
monocytes, or lymphocytes (29). As shown in Fig. 4, administration 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 exhibited significantly less (P < 0.01) aspirin-induced gastric damage (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 combination 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.

Fig. 4. ASA-induced induction of COX-2 expression in the stomach of normal and neutropenic rats. Oral administration of ASA (30 mg/kg) resulted in a significant (*P < 0.05) increase in COX-2 expression (measured by Western blot analysis) in both normal and neutropenic rats. Western blot analysis was performed on tissue samples taken 3 h after ASA (or vehicle) administration. Lanes on the blot correspond to the groups shown on the x-axis of the graph. Each bar represents the mean ± SE of 5 rats per group. *P < 0.05 vs. the corresponding group not treated with ASA.

Fig. 5. ASA-triggered lipoxin A4 [15(R)-epi-LXA4] synthesis in rats given single doses of ASA (30 mg/kg) or NCX-4016 (54 mg/kg) (A) or given these compounds each day for 5 days (B). Each bar represents the mean ± SE of 5–6 rats. *P < 0.05, **P < 0.01 compared with the vehicle-treated group; ***P < 0.01 vs. the corresponding group treated with ASA or NCX-4016 alone.

Fig. 6. A: gastric damage in rats given single doses of ASA (30 mg/kg) or NCX-4016 (54 mg/kg) or given these compounds each day for 5 days. Each bar represents the mean ± SE of 6 rats. *P < 0.05 compared with the single-dose ASA-treated group. B: effects of cotreatment with celecoxib (10 mg/kg ip) on gastric damage in rats given ASA (30 mg/kg) or NCX-4016 (54 mg/kg) each day for 5 days. Celecoxib (or vehicle) was administered at the same time as the final dose of ASA or NCX-4016. Each bar represents the mean ± SE of 5 rats. *P < 0.05 vs. the group treated with ASA alone.
neutrophil in gastric COX-2 expression was attributable to the increase in related to the ability of this drug to release NO, because NO suppressed gastric PG synthesis by NCX-4016, we anticipated an increase in ATL formation. Although a single dose of NCX-4016 did not result in an increase in gastric ATL formation, daily administration of this compound for 5 days did result in a significant 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**

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