A ROLE FOR NEUTROPHILS IN the pathogenesis of ischemia-reperfusion injury in several tissues, including the stomach and small intestine, is supported by considerable evidence (1, 3, 10, 20, 21). Adherence of neutrophils to the vascular endothelium occurs during reperfusion and contributes to disturbed blood flow during this period (12). Moreover, oxygen-derived free radicals and proteases released from activated neutrophils contribute to the tissue injury that occurs after ischemia-reperfusion (10). Depletion of neutrophils with antineutrophil serum (ANS) (21) or prevention of neutrophil adherence and activation (14, 18) is possible that such a derivative of aspirin (NCX-4016) would exert inhibitory effects on neutrophil adherence and therefore be capable of protecting the stomach against shock-induced gastric damage. This hypothesis was tested in this study. Oral administration of NCX-4016 or glyceryl trinitrate or depletion of circulating neutrophils with antineutrophil serum significantly reduced the extent of gastric damage induced by hemorrhagic shock, whereas aspirin had no effect. NCX-4016 and antineutrophil serum pretreatment resulted in significant preservation of gastric blood flow during the shock period. Moreover, NCX-4016, but not aspirin, was capable of inhibiting N-formyl-Met-Leu-Phe-induced leukocyte adherence to postcapillary mesenteric venules. These results suggest that an NO-releasing aspirin derivative reduces the susceptibility of the stomach to shock-induced damage through inhibitory effects on neutrophil adherence to the vascular endothelium.

ischemia-reperfusion; ulcer; nonsteroidal anti-inflammatory drug inhibits the neutrophil adherence normally observed with these drugs and therefore reduce their ability to cause gastric damage. This hypothesis has now been proven with a number of NO-releasing NSAID derivatives, so-called "NO-NSAIDs" (5, 27, 30, 31). Recently, we characterized the antithrombotic effects of an NO-releasing derivative of aspirin, which was capable of inhibiting platelet aggregation despite having no detectable effect on thromboxane synthesis (27). In addition to having greatly reduced ulcerogenic effects, it is possible that NO-NSAIDs, by virtue of their ability to release NO (27, 31), can exert protective effects in other models of gastrointestinal injury. Indeed, we observed a significant protective effect of one such compound in a rat model of endotoxic shock (25).

In the present study, we have examined the effects of an NO-releasing derivative of aspirin (NCX-4016; Fig. 1) in a rat model of gastric ischaemia-reperfusion injury. Induction of hypovolemic shock, followed by reinfusion of the shed blood, results in the formation of hemorrhagic erosions in the gastric mucosa. As there is good evidence that this damage is to some extent neutrophil dependent (21) and that potent NO donors (e.g., sodium nitroprusside) can reduce the severity of such damage (2), it seemed possible that NCX-4016 may also be capable of protecting the stomach in this model. We also attempted to determine if any beneficial effects of NCX-4016 were attributable to effects on neutrophils.

METHODS

Animals. Male Wistar rats (175-200 g) obtained from Charles River Breeding Farms (Montreal, QC, Canada) were housed in polypropylene cages and fed standard laboratory chow and tap water ad libitum. Before each experiment, the rats were deprived of food, but not water, for 18–20 h. All experimental protocols described in this report were approved by the Animal Care Committee of the University of Calgary and were carried out in accordance with the guidelines of the Canadian Council on Animal Care.

Hemorrhagic shock. Groups of rats (n = 5 or more) were orally pretreated with aspirin (10, 50, or 100 mg/kg), equimolar doses of NCX-4016, or vehicle. One hour later, an ex vivo gastric chamber was prepared as described previously (26), and hemorrhagic shock was induced. Briefly, the rats were anesthetized with pentobarbital sodium (65 mg/kg ip). The left carotid artery was cannulated and connected to a pressure transducer for systemic blood pressure recording using a multichannel recorder (Linear recorder SR 3310, Grassphetech, Tokyo, Japan). A femoral artery was cannulated for withdrawal and reinfusion of blood. The stomach was exposed by a midline laparotomy, opened by an incision along the greater curvature, and then pinned over a Plexiglas platform and clamped with a Plexiglas cylinder. The experiments consisted
and then placed in a supine position, and a segment of the midjejunum was exteriorized through an abdominal incision. All exposed tissue was covered with saline-soaked gauze to minimize dehydration of the tissue. The mesentery was carefully placed over an optically clear viewing pedestal that allowed for transillumination of a 2-cm² segment of tissue. The temperature of the pedestal was maintained at 37°C. The mesentery was superfused with warmed bicarbonate-buffered saline (pH 7.4). An intravital microscope (Leitz Wetzlar L25/0.35) and ×10 eyepiece were used to observe the mesenteric microcirculation. A video camera mounted on the microscope projected the image onto a color monitor, and the images were recorded for playback analysis using a videocassette recorder.

Leukocyte adherence was quantified from the videotaped images of the vessels made over a 5-min period beginning at 20 min and at 5 min before and at 15, 30, 45, and 60 min after the start of superfusion of the vessel with N-formyl-Met-Leu-Phe (fMLP) (5 µM; 37°C). All such analyses were performed by an observer who was unaware of the treatments the rats had received. Leukocytes were considered to be “adherent” if they remained stationary for 30 s or more. Rats were pretreated orally 1 h before the start of these experiments with aspirin (50 mg/kg), an equimolar dose of NCX-4016 (93 mg/kg), or vehicle.

Platelet guanosine 3',5'-cyclic monophosphate concentrations. The effects of aspirin and NCX-4016 on platelet concentrations of guanosine 3',5'-cyclic monophosphate (cGMP) were determined as described previously (27). Briefly, blood was drawn from the carotid artery of rats anesthetized with pentobarbital sodium (60 mg/kg ip). Platelet-rich plasma (2 × 10⁶ platelets/ml) was prepared, and aliquots (0.4 ml) were added to Eppendorf tubes containing aspirin (1–1,000 µM), NCX-4016 (1–1,000 µM), sodium nitroprusside (100 µM), or vehicle (methanol). The experiment was performed in triplicate. The tubes were placed in a shaking water bath (37°C) for 10 min and then centrifuged for 20 s at 1,000 g. The supernatant was removed and discarded. The pellet was resuspended in 100 µl of ice-cold phosphate-buffered saline (pH 7.4) and then sonicated for 20 s. After centrifugation (20 s, 1,000 g), the supernatants were stored at −20°C until the assay for cGMP was performed. A commercially available enzyme-linked immunosorbent assay (ELISA) kit for cGMP was used.

Materials. Aspirin, naproxen, and fMLP were obtained from Sigma Chemical (St. Louis, MO). NCX-4016 (2-acetoxy-benzoate 2-[1-nitroxy-methyl]-phenyl ester) was provided by NicOx (Paris, France). Glyceryl trinitrate was obtained from Omega (Montreal, QC, Canada). ANS was obtained from Accurate Chemical and Scientific (Westbury, NY). The stock solution was diluted 10-fold in sterile 0.9% saline, and 2 ml were administered to the rat at 24 h and at 1 h before beginning each experiment. The cGMP ELISA kit was obtained from Caymen Chemical (Ann Arbor, MI). All other reagents were obtained from VWR (Edmonton, AB, Canada).

Statistical analysis. All data are expressed as means ± SE. Comparisons among groups of data were made using an analysis of variance followed by a Student-Newman-Keuls test. An associated probability (P value) of <5% was considered significant.

RESULTS

Hemorrhagic shock-induced gastric damage. During the period of reinfusion of shed blood, hemorrhagic lesions began to appear in the corpus region of the stomach. By the end of the experiment, vehicle-pretreated rats exhibited damage to approximately
one-half of the glandular mucosa (Fig. 2). Pretreatment with NCX-4016 resulted in a significant reduction in the extent of hemorrhagic damage. All three doses of NCX-4016 were effective. In contrast, aspirin did not significantly affect the extent of gastric damage at any of the doses tested. The resting systemic arterial blood pressure was not significantly affected by pretreatment with aspirin or NCX-4016.

Pretreatment with glyceryl trinitrate 1 h before induction of shock did not significantly alter the susceptibility of the stomach to damage induced by hemorrhagic shock (Fig. 3). On the other hand, administration of glyceryl trinitrate during the period of shock resulted in a significant (P < 0.05) reduction in the extent of gastric damage. Similarly, pretreatment with ANS resulted in a marked decrease (P < 0.05) in the extent of gastric damage.

Gastric blood flow. Induction of hemorrhagic shock resulted in a profound decrease in gastric blood flow (Fig. 4). In vehicle-treated rats, decreasing the systemic arterial blood pressure to 25 mmHg resulted in a rapid decline in gastric blood flow to only 3% of basal levels. Pretreatment with aspirin did not alter the decrease in blood flow during shock. However, pretreatment with NCX-4016 or ANS resulted in significant preservation of gastric blood flow during the shock period. The return of blood flow to the stomach during the reinfusion of shed blood was similar in all four experimental groups.

Leukocyte adherence. Under basal conditions, a few leukocytes were observed adhering to postcapillary mesenteric venules (Fig. 5). Pretreatment with NCX-4016 did not affect basal leukocyte adherence. However, pretreatment with aspirin resulted in a significant increase (~5-fold) in the number of leukocytes adhering to the vessel wall at the beginning of the experiment. Superfusion of the postcapillary mesenteric venules with fMLP resulted in a significant increase in leukocyte adherence in the vehicle-treated rats. The number of leukocytes adhering to the vessel wall increased to a similar level in rats treated with aspirin. However, in rats pretreated with NCX-4016, there was significantly less (P < 0.05) leukocyte adherence during superfusion with fMLP than was observed in the other two groups.
Platelet cGMP concentrations. Incubation of rat platelets in the presence of aspirin (1–1,000 µM) did not significantly affect cGMP concentrations relative to those observed in platelets incubated only with vehicle (Fig. 6). However, incubation of platelets in the presence of NCX-4016 resulted in a concentration-dependent increase in cGMP levels. Sodium nitroprusside (100 µM) also markedly elevated platelet cGMP concentrations (Fig. 6).

**DISCUSSION**

NO has well-documented protective effects in the stomach (13, 16, 17), and suppression of endogenous NO synthesis renders the stomach more susceptible to injury induced by topical irritants (32). The mechanism through which NO is capable of protecting the stomach is not clear, although it has been shown to be important in the maintenance of gastric mucosal blood flow, as a stimulant of mucus secretion, and as an inhibitor of neutrophil adherence to the vascular endothelium (24). The development of NO-releasing NSAIDs was based on the premise that adherence of neutrophils to the vascular endothelium is a critical step in the pathogenesis of NSAID-induced gastric damage, so the NO release from these compounds should prevent neutrophil adherence and in turn prevent gastric mucosal injury (23). As hemorrhagic shock-induced gastric damage has also been shown to be, at least in part, a neutrophil-dependent process (21), the present study was performed to evaluate whether or not an NO-releasing aspirin derivative (NCX-4016) would be capable of conferring protection to the gastric mucosa. The results clearly demonstrate that this compound can reduce the severity of hemorrhagic shock-induced gastric damage. A similar effect could be achieved by depleting the rats of circulating neutrophils (with an ANS), suggesting that it was through inhibition of neutrophil adherence or activation that NCX-4016 produced its effects. Also consistent with this hypothesis are the observations that both NCX-4016 and ANS increased gastric blood flow during the shock period. This is consistent with the previous observations of Smith et al. (21) that depletion of circulating neutrophils results in significant preservation of gastric blood flow and also with the findings of Jerome et al. (12) that prevention of neutrophil adherence with an anti-CD18 antibody results in improved tissue blood flow after shock. Finally, the ability of NCX-4016 to inhibit adherence of leukocytes to the vascular endothelium was confirmed in the intravital microscopy studies.

Reduction of the severity of ischemia-reperfusion-induced gastric damage by NO-generating drugs, such as sodium nitroprusside, was previously demonstrated by Andrews et al. (2). In the present study, administration of glyceryl trinitrate 1 h before induction of shock failed to significantly affect the severity of damage, whereas administration of this NO donor during the period of shock significantly reduced gastric damage. We selected the 5 mg/kg dose because it was sufficiently high to produce a decrease in systemic arterial blood pressure, indicating that NO had been generated, but sufficiently low so as not to produce a prolonged depression of blood pressure that might itself render the mucosa more susceptible to injury. On the other hand, the dose of NCX-4016 given did not significantly alter systemic blood pressure. Our interpretation of these data is that the time and rate of delivery of NO to the gastric mucosa are likely to be important factors in determining whether a compound can produce a beneficial effect. Glyceryl trinitrate is absorbed and generates NO very quickly, as was evident by the decrease in systemic blood pressure within seconds of its administration. To observe a protective effect of this compound, it was necessary to administer the compound during, rather than 1 h before, the period of shock. Similar to other NO-NSAIDs, NCX-4016 has been shown to release NO (4) and to inhibit platelet aggregation (29). In the present study, we also observed that NCX-4016 stimulated a significant increase in cGMP levels within platelets during a 10-min incubation period, consistent with the generation of NO. In contrast, we have previously reported that administration of sodium nitroprusside at a dose that produced profound hypotension did not significantly alter platelet aggregation (27). Thus it appears that it is the slow release of NO from NO-NSAIDs that accounts for their inhibitory effects on platelet aggregation and probably also for their beneficial effect in the gastrointestinal tract. Also consistent with this hypothesis are the observations of Lopez-Belmonte et al. (16) that very low doses of glyceryl trinitrate could reduce the susceptibility of the stomach to damage but that larger doses actually exacerbate damage.

As mentioned above, NCX-4016 has been shown to have inhibitory effects on platelet aggregation (4). The role of platelets in the pathogenesis of hemorrhagic shock-induced gastric damage has not, to our knowledge, been investigated. However, it is unlikely that
inhibition of platelet aggregation accounted for the beneficial effects of NCX-4016 in the present study, since aspirin is as potent an inhibitor of platelet aggregation as NCX-4016 (4) but did not confer any protective effect. Moreover, we found that depletion of circulating platelets with an antiplatelet serum did not reduce the susceptibility of the stomach to damage induced by hemorrhagic shock (unpublished data).

The results of the present study suggest that NCX-4016 reduced gastric damage through inhibitory effects on neutrophil adherence to the vascular endothelium during the shock period. As a result, there was significant preservation of blood flow to the stomach. We have previously demonstrated that an increase in gastric blood flow during shock of the same magnitude as was observed in rats pretreated with NCX-4016 or ANS is sufficient to prevent much of the gastric damage normally seen in hemorrhagic shock (26). NO donors have been shown to inhibit neutrophil adherence (8), and several mechanisms have been proposed to explain this effect, including scavenging of superoxide anion (a proadhesive factor) (8) and inhibition of the expression of the proinflammatory nuclear transcription factor NF-κB (6). On the other hand, NO could conceivably inhibit ischemia-reperfusion-induced neutrophil adherence through inhibitory effects on platelet-activating factor (PAF) release. PAF receptor antagonists have been shown to dose dependently reduce the severity of hemorrhagic shock-induced gastric damage in the rat and to cause a similar preservation of gastric blood flow during the shock period as was observed in the present study (26). PAF has been shown to be expressed on the luminal surface of endothelial cells during hypoxia and has been suggested to play a key role in ischemia-associated neutrophil adherence (15, 33). NO has been shown to inhibit PAF release from endothelial cells (9) and mast cells (11). Direct effects of NO-NSAIIDs, including NCX-4016, on PAF release have not been reported.

In summary, the present study demonstrates that an NO-releasing derivative of aspirin (NCX-4016), which has been shown to spare the stomach of damage normally associated with aspirin (29), is capable of reducing the severity of hemorrhagic shock-induced gastric damage. It seems likely that these beneficial effects of NCX-4016 are attributable to its inhibitory effects on neutrophil adherence to the vascular endothelium. Compounds that release NO at a slow rate may have utility for treatment of conditions in which the gastrointestinal tract is susceptible to ischemia-reperfusion injury.

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REFERENCES


