Effect of Nitric Oxide-Releasing Aspirin Derivative On Gastric Functional and Ulcerogenic Responses in Rats: Comparison With Plain Aspirin

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ABSTRACT
The effects of a nitric oxide (NO)-releasing derivative of aspirin, NCX-4016, on gastric functional and ulcerogenic responses in rat stomachs were examined in comparison with those of aspirin. Topical application of aspirin (80 mM) to the stomach markedly decreased transmucosal potential difference and slightly increased luminal pH (acid back-diffusion) with minimal effect on mucosal blood flow, whereas NCX-4016 caused a marked increase in mucosal blood flow with no effect on potential difference and pH. Aspirin itself was ulcerogenic, causing damage in the mucosa when administered p.o., and it markedly potentiated gastric ulcerogenic response to hypothermic stress (28°C–30°C) with no effect on acid secretion when given s.c. NCX-4016, however, was not ulcerogenic by itself, did not modify the ulcerogenic response to stress and even showed a dose-dependent protection against HCl/ethanol-induced gastric lesions. When NCX-4016 was given intragastrically to pylorus-ligated rats, a large amount of NO was detected in both gastric contents and serum. NCX-4016 administered either p.o. or s.c. produced an equipotent inhibition of mucosal PGE2 generation in the stomach, as compared with aspirin. In addition, both aspirin and NCX-4016 suppressed carrageenan-induced rat paw edema. These results suggest that, unlike aspirin, the NO-releasing derivative of aspirin NCX-4016 neither had a topical irritating action on the stomach nor exerted a worsening effect on gastric ulcerogenic response to stress, but rather provided gastric protection against ethanol, despite inhibiting cyclo-oxygenase activity and showing anti-inflammatory action much as aspirin does. NCX-4016, probably by releasing NO, exerted protective effects that counteracted the potential damaging effects of cyclo-oxygenase inhibition.

ASA, a NSAID, causes damage in the stomach as an unwanted side effect (Levy, 1974; Lanza, 1984). There are two major components to the ulcerogenic effects of ASA in the stomach: the topical irritant action on the epithelium and the suppression of PG biosynthesis (Davenport, 1969; Vane, 1971; Okabe et al., 1974). Several components of gastric mucosal defense are influenced or mediated by PGs, including mucus and bicarbonate secretion, blood flow, epithelial cell turn over and repair and mucosal immunocyte function (Robert, 1984). Therefore, it is possible that inhibition of PG biosynthesis leads to a reduction in the ability of the gastric mucosa to defend itself against luminal irritants.

Attempts to develop NSAIDs that spare the GI tract from injury have produced selective inhibitors of COX-2, NO-releasing NSAIDs and NSAIDs preassociated with zwitterionic phospholipids (Putaki et al., 1994; Wallace et al., 1994a; Lichtenberger et al., 1995; Wallace, 1997). It is of interest that the coupling of a NO-releasing moiety to standard NSAIDs markedly reduced their short-term ulcerogenic properties without altering their effectiveness as anti-inflammatory drugs or COX inhibitors (Wallace et al., 1994b; 1995a; Davies et al., 1997). The rationale behind this strategy is that the NO released from these derivatives will exert beneficial effects on the mucosa by modulating gastric functions such as mucosal blood flow. The NO-releasing ASAs such as NCX-4215 and NCX-4016 are similarly derived from ASA and have been shown to exhibit antithrombotic activity comparable to that of ASA with less damage in the gastric mucosa (Wallace et al., 1995b; Lechi et al., 1996). However, the mechanism responsible for the reduced ulcerogenic property of the NO-releasing derivatives of ASA has not yet been elucidated.

In the present study, we examined the effects of a NO-releasing derivative of ASA (NCX-4016; fig. 1) on transmucosal PD, GMBF and mucosal responses to ulcerogenic stimulation in the rat and compared those effects with the effects of its parent compound, ASA.

Materials and Methods
Male Sprague-Dawley rats weighing about 200 to 230 g (Charles River, GS, Yokohama, Japan), were used in all experiments. The

ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; ASA, aspirin; NO, nitric oxide; PG, prostaglandin; COX, cyclooxygenase; PD, potential difference; GMBF, gastric mucosal blood flow; CMC, carboxymethylcellulose.
animals were kept in individual cages with raised mesh bottoms and deprived of food but allowed free access to tap water for 18 hr before the experiments. Studies were carried out using 5 to 7 rats under urethane-anesthetized conditions (1.25 g/kg), unless otherwise specified.

**Determination of Gastric PD, pH and Mucosal Blood Flow**

The methods used for measuring transmucosal PD, luminal pH and GMBF have been described previously (Matsumoto et al., 1992; Takeuchi et al., 1994). In brief, the abdomen was opened through a midline incision, and the stomach was exposed, mounted on an ex vivo chamber (exposed area 3.14 mm²) and perfused at a flow rate of 1 ml/min with saline that was suffused with 100% O₂ and kept in a reservoir. The pH of fluid emerging from the chamber was measured using a flow-type pH glass electrode (6901-25T, Horiba, Kyoto, Japan) and by placing a probe gently on the surface of the corpus mucosa using a balancer (Medical Agent, Kyoto, Japan). After PD, pH and GMBF had stabilized, the perfusion system was interrupted, the solution in the chamber was withdrawn. The mucosa was rinsed with saline, and the area (square millimeters) of each lesion developed in the glandular mucosa was measured under a dissecting microscope. ASA (20–100 mg/kg) or NCX-4016 (33–165 mg/kg) was administered s.c. 30 min before the onset of hypothermia. In some cases, the effect of vagotomy on these responses was also examined to confirm the vagal dependence of the ulcerogenic response to stress. Vagotomy was performed bilaterally at the cervical portion 1 hr before the onset of hypothermia.

**Study B.** The animals were exposed to cold without any surgical manipulation. In brief, the animal was placed in a styrofoam box, and the body temperature was lowered between 28°C and 30°C with a refrigerant pack (Niida et al., 1991a). Rectal temperature was monitored continuously with a rectal thermometer (Nihon Koden, MGA-3, Tokyo, Japan). At the end of 4-hr experiments, the stomachs were removed, and the area (square millimeters) of each lesion developed in the glandular mucosa was measured under a dissecting microscope. ASA (20 mg/kg) or NCX-4016 (33 mg/kg) was administered s.c. 30 min before the onset of hypothermia. In some cases, the effect of FK409, a NO donor (Kita et al., 1994), on HCl/ethanol-induced gastric lesions was examined. FK409 (1 mg/kg) was given p.o. 30 min before HCl/ethanol treatment.

**Measurement of Gastric Acid Secretion**

Effects of ASA and NCX-4016 on gastric acid secretory response to thermic stress were examined under urethane-anesthetized conditions. Acid secretion was measured in the acute fistula rat according to a previously published method (Niida et al., 1991b). Briefly, the abdomen was incised, and both the stomach and the duodenum were exposed. An acute fistula (I.D. 3 mm), made with a polyethylene tube, was inserted into the stomach from a small incision made in the duodenum and was held in place by a ligature around the pylorus. Then the body temperature was maintained at 28°C to 30°C as described above. The stomach was filled with 2 ml of saline (154 mM NaCl) through the fistula, and the solution was changed every 15 min. The collected samples were centrifuged at 3000 rpm for 15 min and titrated to pH 7.0 against 0.1 N NaOH using an autoburette (Hiranuma Coomite-S, Tokyo, Japan). ASA (20 mg/kg) or NCX-4016 (33 mg/kg) was given s.c. 30 min before the onset of hypothermia.

**Formation of Paw Edema by Carrageenan**

Paw edema was induced in unanesthetized rats by subplantar injection of carrageenan (0.1 ml of 1% carrageenan-saline solution) into the right hind paw (Salvemini et al., 1996). Paw volume was measured using a plethysmometer immediately before the injection of carrageenan and thereafter at 2-hr intervals for 6 hr. Edema was expressed as the increase in paw volume (milliliters) after carrageenan injection relative to the preinjection value for each animal. ASA (20 mg/kg) or NCX-4016 (33 mg/kg) was given s.c. 30 min before carrageenan injection.

**Determination of PGE₂**

The effects of ASA and NCX-4016 on mucosal PGE₂ contents in the stomach were examined under normal conditions. The animals were given ASA (20 mg/kg) or NCX-4016 (33 mg/kg) p.o. or s.c. and were killed 2 hr later. The stomachs were removed, and the corpus mucosa was isolated, weighed, and put in a tube containing 100% ethanol plus 0.1 M indomethacin (Putaki et al., 1993). Then the
samples were minced by scissors, homogenized and centrifuged for 10 min at 12,000 rpm at 4°C. The supernatant of each sample was used for determination of PGE₂ by EIA via a PGE₂-kit (Cayman Chemical Co., Ann Arbor, MI).

**Measurement of NOx in Gastric Contents and Serum**

NOx levels were determined in both the gastric contents and the serum of pylorus-ligated rats after p.o. administration of NCX-4016. Under ether anesthesia, the abdomen was incised, and the pylorus was ligated. Ninety minutes after the ligation, blood was collected from the descending aorta, and the gastric contents were recovered. Samples were centrifuged for 15 min at 3000 rpm and stored at −80°C until the assay. NOx was measured in aliquots of the samples by the Griess method after reduction of nitrate to nitrite with nitrate reductase (from Aspergillus; Sigma Chemicals, St. Louis, MO). Nitrites were incubated with Griess reagent (0.1% naphthylene diamine dihydrochloride and 1% sulfanilamide in 2.5% H₃PO₄) for 10 min at room temperature, and the absorbance at 550 nm was measured.

**Preparation of Drugs**

Drugs used in this study were urethane (Tokyo Kasei, Tokyo, Japan), ASA and carrageenan (Sigma) NCX-4016 (NicOx, Paris, France) and FK409 (Fujisawa, Osaka, Japan). ASA or NCX-4016 was suspended in 1% CMC; other agents were dissolved in saline. Each agent was prepared immediately before use and administered i.p., s.c. or p.o. in a volume of 0.5 ml per 100 g b.wt. or applied topically to the chamber in a volume of 2 ml per rat.

**Statistics**

Data are presented as the means ± S.E. from 5 to 7 rats per group. Statistical analyses were performed using a two-tailed Dunnett’s multiple comparison test, and values of P < .05 were regarded as significant.

**Results**

**Effects of ASA and NCX-4016 on gastric PD, pH and GMBF.** A chambered rat stomach generated PD of −30 to 35 mV (mucosa negative), secreted acid at rates of about 2 to 3 μEq/5 min as basal secretion to maintain luminal pH of 3.6 to 3.8 and showed a relatively constant GMBF of about 80 to 100 mV² (arbitrary unit). Topical application of 80 mM ASA for 30 min caused a marked reduction in PD from −32.4 ± 1.8 mV to −13.1 ± 1.2 mV (∆PD: 19.7 ± 2.8 mV²), followed by an increase of luminal pH (∆pH: 1.2 ± 0.2) (fig. 2). After ASA treatment, acid secretion was also decreased from 2.6 ± 0.2 μEq/5 min to values of <1 μEq/5 min within 60 min and remained lowered during a 2 hr-test period (not shown). GMBF was slightly increased immediately after exposure of the mucosa to ASA but returned to or below base-line values during the exposure. On the other hand, the mucosal exposure to NCX-4016 (80 mM) had no effect on either PD or luminal pH but caused a marked elevation in GMBF. The GMBF was significantly elevated during exposure to NCX-4016, reaching a peak increase of 82.1 ± 11.7%, and remained significantly elevated for 30 min even after removal of NCX-4016 from the chamber (fig. 2C).

**Effects of ASA and NCX-4016 on gastric mucosa.** The p.o. administration of ASA (80 mM; 72 mg/kg), suspended in CMC, caused damage in the gastric mucosa consisting of both nonhemorrhagic and hemorrhagic lesions, the total lesion score being 8.4 ± 2.3 mm² (fig. 3). The ulcerogenic action of ASA was markedly increased when ASA was administered together with 50 mM HCl. The damage consisted largely of hemorrhagic lesions, the total lesion score being 28.1 ± 6.2 mm². On the other hand, no lesion was observed in the stomach when NCX-4016 (80 mM; 119 mg/kg) was given p.o., either in the absence or in the presence of 50 mM HCl.

**Effects of ASA and NCX-4016 on gastric acid secretory and ulcerogenic responses to stress.** When the animals were exposed to ambient temperatures lowered with a refrigerator pack, the body temperature could be well maintained in the range of 28°C to 30°C during a 4 hr-test period. Lowering of the body temperature produced a marked increase of acid secretion and hemorrhagic damage in the stomach. The acid output was increased from 78.7 ± 4.7 μEq/4 hr to 428.1 ± 68.3 μEq/4 hr, and the lesion score was 17.3 ± 3.1 mm² (fig. 4, A and B). These responses were vagally mediated and were almost totally inhibited by bilateral vagotomy. Pre-treatment of the animals with ASA (20 mg/kg s.c.) signifi-
cantly increased the severity of gastric lesions without having any effect on the acid secretory response, the lesion score being 39.2 \pm 8.1 \text{ mm}^2. \text{ On the other hand, prior administration of NCX-4016 (33 mg/kg) did not significantly affect either the acid secretory or the ulcerogenic response to hypothermic stress: the lesion score was 12.4 \pm 2.8 \text{ mm}^2, which is significantly less than that observed in the presence of ASA.}

**Fig. 4.** Effects of ASA and NCX-4016 on gastric acid secretory (panel A) and ulcerogenic (panel B) responses induced by hypothermic stress in anesthetized rats. The animals were placed in a styrene foam box, and the body temperature was maintained between 28°C and 30°C for 4 hr by exposing the animals to a refrigerant pack. ASA (20 mg/kg) or NCX-4016 (33 mg/kg) was given s.c. 30 min before the onset of stress. Data are presented as the means \pm S.E. from 5 to 7 rats. Statistically significant difference at P < .05; *from normal (36°C), #from control, \#from ASA.

**Effects of ASA and NCX-4016 on HCl/ethanol-induced gastric lesions.** Intragastric administration of HCl/ethanol (60% in 150 mM HCl) caused multiple band-like lesions in the gastric mucosa, the lesion score being 148.2 \pm 19.6 \text{ mm}^2. \text{ The severity of these lesions was dose-dependently reduced by prior p.o. administration of NCX-4016 (33–165 mg/kg), and a significant effect was observed at 33 mg/kg or greater, the inhibition at 119 mg/kg being 59.3% (fig. 5). Pretreatment of the animals with ASA also reduced the severity of the lesions, but this effect was significant only at 100 mg/kg. On the other hand, gastric damage in response to HCl/ethanol was also prevented by an exogenous NO donor, FK409 (1 mg/kg), the inhibition being 91.2%.

**Fig. 5.** Effects of ASA and NCX-4016 on HCl/ethanol-induced gastric lesions in rats. The animals were administered 1 ml of HCl/ethanol (60% in 150 mM HCl) and were killed 1 hr later. ASA (20 \sim 100 \text{ mg/kg}), NCX-4016 (33 \sim 165 \text{ mg/kg}) or FK409 (1 \text{ mg/kg}) was administered p.o. 30 min before HCl/ethanol treatment. Data are presented as the means \pm S.E. from 5 to 7 rats. *Statistically significant difference from controls, at P < .05.

**Effects of ASA and NCX-4016 on PGE_2 levels in gastric mucosa.** Levels of PGE_2 in the normal rat gastric mucosa were 221.1 \pm 24.2 \text{ ng/g tissue}. ASA given either p.o. or s.c. significantly reduced the PGE_2 levels in the corpus mucosa at 20 mg/kg, the inhibition being 88.7% and 73.2%, respectively (fig. 6). The mucosal PGE_2 levels were also significantly reduced by NCX-4016 (33 mg/kg), irrespective of the route of administration, the inhibition being 68.7% and 50.1%, respectively.

**Fig. 6.** Effects of ASA and NCX-4016 on gastric mucosal PGE_2 contents in rat stomachs. The animals were given ASA (20 mg/kg) or NCX-4016 (33 mg/kg) either p.o. or s.c. and were killed 2 hr later. The levels of PGE_2 were determined in the corpus mucosa using EIA. Data are presented as the means \pm S.E. from 5 to 8 rats. *Statistically significant difference from controls, at P < .05.
ASA produces gastric lesions when administered p.o. but not when administered parenterally, the former effect is more crucial in causing gastric mucosal damage (Ligumsky et al., 1983). In fact, topical irritant properties may be related to the ability of ASA to decrease the hydrophobicity of the mucus gel layer in the stomach, which has been suggested to be a primary barrier to damage induced by acid (Goddard et al., 1987). In the present study, the mucosal application of ASA produced a reduction of PD followed by a small increase of luminal pH. The latter effect may be mainly attributable to a passive diffusion of HCO$_3^-$ ion as a result of barrier disruption. However, NCX-4016, a NO-releasing derivative of ASA, applied topically to the stomach had no effect on PD and pH, which suggests a lack of direct irritating action on the mucosa. It should also be noted that NCX-4016 produced a sustained increase of GMBF after the mucosal application, whereas ASA increased GMBF temporarily immediately after application. In agreement with previous findings (Wallace et al., 1994b; 1995a; 1995b; Davies et al., 1997), we observed that the addition of a nitroxybutylester group to NSAID markedly reduced its short-term ulcerogenic property without altering its effectiveness as a COX inhibitor. Intragastric administration of NCX-4016 did not produce any damage in the stomach even in the presence of exogenous HCl, despite decreasing the mucosal PGE$_2$ contents, although ASA decreased the mucosal PGE$_2$ biosynthesis and caused hemorrhagic damage in the stomach. Wallace et al., (1995b) reported that a NO-releasing derivative of ASA (NCX-4215) inhibited PG synthesis in platelets but not in the gastric mucosa. The reason for the different results remains unknown, but different experimental conditions may explain it; they measured 6-keto PGF$_{1alpha}$ using gastric tissue samples and adding the NO-releasing ASA NCX-4215 in vitro, whereas we measured PGE$_2$ using tissue samples taken from animals pretreated with the NO-releasing ASA NCX-4016.

The mechanism responsible for the NO-releasing ASA derivatives, lacking the ulcerogenic property is not yet clear. One possibility is that the topical irritant property was reduced by adding a nitroxy-butyl moiety to the ASA, therefore reducing the ulcerogenic effect.

Not only are NSAIDs by themselves ulcerogenic in the stomach, but they also potentiate the ulcerogenic response to various stimuli, including stress (Whittle, 1983; Konturek et al., 1990; Hirata et al., 1997). Konturek et al. (1990) reported that gastric lesions induced by water-immersion stress were markedly worsened by indomethacin at a low dose that did not cause any damage in the stomach. We observed in the present study that s.c. pretreatment with ASA markedly increased the gastric ulcerogenic response to hypothermic stress without altering the acid secretory response. Because ASA given parenterally decreased the mucosal PGE$_2$ generation but did not cause damage in the stomach (Ligmusky et al., 1983), the aggravating effect on stress ulcers may be related to a deficiency of endogenous PGs. As evidenced in this study, NCX-4016 did not potentiate the gastric ulcerogenic response to stress, despite inhibiting PG generation in the stomach. The different results cannot be explained by the presence or absence of topical irritating action, because both ASA and NCX-4016 were given parenterally before the onset of stress. These results suggest that NCX-4016, by releasing NO, exerted a protective effect that counteracted the potential damaging effects of COX inhibition. Indeed, we found a

**Discussion**

One recently devised approach to developing NSAIDs with GI-sparing properties is the coupling of an NO-releasing moiety to standard NSAIDs (Wallace et al., 1994b; 1995a; 1995b; Wallace, 1997; Davies et al., 1997). The rationale behind this strategy is that the NO released from the compounds will exert beneficial effects on the gastric mucosa by enhancing the mucosal defensive ability (Whittle et al., 1990; Moncada et al., 1991). This contention has been supported by previous studies using several NO-releasing derivatives of NSAIDs, such as nitrofenac, NO-naproxen and NO-flubiprofen, in spite of the fact that these agents showed equipotent anti-inflammatory actions similar to patent NSAIDs (Wallace et al., 1994b; 1995a,b; Davies et al., 1997). In the present study, we had similar results using a NO-releasing derivative of ASA, NCX-4016, and further showed that this compound, devoid of a direct irritating action on the stomach, was not ulcerogenic but rather exhibited gastric cytoprotection against HCl/ethanol-induced injury.

There are two major components of the ulcerogenic effects of ASA in the stomach: the topical irritant effect on the epithelium and the suppression of PG biosynthesis. Because
considerable amount of NOx, the metabolites of NO, in the lumen of the stomach as well as in the serum after p.o. administration of the NO-releasing derivative of ASA. It should be noted that a NO-releasing ASA (NCX-4215) did not alter systemic arterial blood pressure when administered i.v. to the rat, despite releasing NO (Wallace et al., 1995b). Furthermore, we found that NCX-4016 conferred a dose-dependent protection against HCl/ethanol-induced gastric damage. Although ASA also provided protection against HCl/ethanol, this action was weak and was not dose-dependent. Because a potent inhibition of HCl/ethanol-induced lesions was observed on administration of an exogenous NO donor, FK409 (Kita et al., 1994), the protective effect of NCX-4016 may be attributable to the NO released from this compound. The reason why ASA exhibited a weak protective effect remains unknown but may be associated with luminal dilution of the irritant as a result of barrier disruption.

The mechanism through which NO protects the gastric mucosa against damage is not entirely clear. It is now well established that NO is an important mediator of gastric mucosal defense, modulating mucosal blood flow and mucus secretion (MacNaughton et al., 1989; Whittle et al., 1990; Moncada et al., 1991). Because NCX-4016 applied topically to the stomach caused a persistent increase of GMBF, this effect may contribute to the protective action of this compound. In addition, recent studies showed the important role of neutrophils in the pathogenesis of ASA-induced gastric lesions (Wallace et al., 1990; Yoshida et al., 1993; Andrews et al., 1994). NO inhibits neutrophil activation and scavenges oxygen metabolites (McCall et al., 1988), so it is possible that this molecule interferes with the neutrophil-related process in tissue injury. Indeed, a recent study showed that this particular NO-releasing derivative of ASA, NCX-4016, inhibited leukocyte adherence to the vascular endothelium and prevented gastric damage induced by hemorrhagic shock (Wallace et al., 1997b). The possible mechanisms through which NO protects the gastric mucosa require further study.

On the other hand, a potent anti-inflammatory action against carrageenan-induced paw edema was produced by NCX-4016 as well as by the parent compound ASA. Because intraperitoneal injection of carrageenan produces an increase of PGE2 production and induction of de novo synthesis of COX in pleural exudate cells (Masferrer et al., 1994), NCX-4016 suppressed carrageenan-induced paw edema by inhibiting COX enzymatic activity in inflammatory cells. This observation suggests that NCX-4016 inhibits PG synthesis in the gastric mucosa and at a peripheral site of inflammation as effectively as ASA.

Taken together, the results of the present study show that unlike ASA, the NO-releasing ASA derivative NCX-4016 is totally devoid of topical irritant action, is not ulcerogenic, does not potentiate gastric ulcerogenic response to stress and is rather protective of the stomach, all of which can be attributed to an anti-inflammatory mechanism. Thus we conclude that adding a nitroxybutylester group to ASA markedly reduces its ulcerogenic property without altering its effectiveness as an anti-inflammatory agent and COX inhibitor.

References


Wallace JL, Reuter B, Del Soldato P, Baylouny AR and Cirino G (1995b) Anti-

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