A novel small molecule CFTR inhibitor attenuates HCO$_3^-$ secretion and duodenal ulcer formation in rats

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A novel small molecule CFTR inhibitor attenuates HCO$_3^-$ secretion and duodenal ulcer formation in rats. Am J Physiol Gastrointest Liver Physiol 289: G753–G759, 2005. First published May 19, 2005; doi:10.1152/ajpgi.00130.2005.—The cystic fibrosis (CF) transmembrane conductance regulator (CFTR) plays a crucial role in mediating duodenal bicarbonate (HCO$_3^-$) secretion (DBS). Although impaired DBS is observed in CF mutant mice and in CF patients, which would predict increased ulcer susceptibility, duodenal injury is rarely observed in CF patients and is reduced in CF mutant mice. To explain this apparent paradox, we hypothesized that CFTR dysfunction increases cellular [HCO$_3^-$] and buffering power. To further test this hypothesis, we examined the effect of a novel, potent, and highly selective CFTR inhibitor, CFTRinh-172, on DBS and duodenal ulceration in rats. DBS was measured in situ using a standard loop perfusion model with a pH stat under isoflurane anesthesia. Duodenal ulcers were induced in rats by cysteamine with or without CFTRinh-172 pretreatment 1 h before cysteamine. Superfusion of CFTRinh-172 (0.1–10 μM) over the duodenal mucosa had no effect on basal DBS but at 10 μM inhibited acid-induced DBS, suggesting that its effect was limited to CFTR activation. Acid-induced DBS was abolished at 1 and 3 h and was reduced 24 h after treatment with CFTRinh-172, although basal DBS was increased at 24 h. CFTRinh-172 treatment had no effect on gastric acid or HCO$_3^-$ secretion. Duodenal ulcers were observed 24 h after cysteamine treatment but were reduced in CFTRinh-172-pretreated rats. CFTRinh-172 acutely produces CFTR dysfunction in rodents for up to 24 h. CFTR inhibition reduces acid-induced DBS but also prevents duodenal ulcer formation, supporting our hypothesis that intracellular HCO$_3^-$ may be an important protective mechanism for duodenal epithelial cells.

CFTRinh-172; duodenal bicarbonate secretion; duodenal ulcer; cysteamine; luminal acid

The proximal duodenal mucosa is unique in that it is the only leaky gastrointestinal epithelium exposed to gastric acid. Because “leaky” intercellular junctions create an incomplete barrier to acid diffusion, nonstructural defense mechanisms such as mucus secretion, bicarbonate (HCO$_3^-$) secretion, and blood flow are of prime importance in the defense from luminal acid (13, 23). These defense mechanisms protect the mucosal cells from the intense acid stress continuously present in the gastroduodenal lumen (36, 42). In the presence of luminal acid, the mucosal epithelial cells will be irreversibly acidified, leading to cellular necrosis (28, 37). The major duodenal defense mechanism is epithelial HCO$_3^-$ secretion, which is thought to neutralize luminal acid before it reaches the mucosal cells (5, 12). Although the mechanism of duodenal HCO$_3^-$ secretion (DBS) is not fully understood, it clearly involves the cystic fibrosis (CF) transmembrane conductance regulator (CFTR), because CFTR absence or dysfunction is associated with low levels of basal and stimulated HCO$_3^-$ secretion (8, 39).

CF is the most common monogenetically transmitted lethal disease among populations of northern European ancestry, causing considerable morbidity and early mortality (41). Although luminal acid-related DBS is impaired in CF knockout mice (19) and in CF patients (39), which would predict increased ulcer susceptibility, duodenal ulceration is rarely observed in CF patients (6, 31). To explain this apparent paradox, we hypothesized that the diminished HCO$_3^-$ exit from the epithelial cells associated with CFTR dysfunction increases cellular [HCO$_3^-$] and hence enhances buffering of cellular H$^+$. Because HCO$_3^-$ uptake or accumulation into the epithelial cells occurs before DBS (3, 11), inhibition of CFTR should augment cellular [HCO$_3^-$] and, hence, buffering power. This concept is supported by our observation that the CI$^-$ channel inhibitor 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) alkalinizes epithelial cells and reduces cellular acidification and injury due to luminal acid, even though NPPB inhibits acid-induced DBS (4). Baseline intracellular pH is higher in duodenoctyes obtained from CF patients (39), also supporting the concept of HCO$_3^-$ “trapping” in cells with defective CFTR function. Furthermore, the duodenum of CF F508 mutant mice is more resistant to cellular acidification and injury in response to luminal acid (17). Nevertheless, we have not yet studied the effect of acute CFTR dysfunction on duodenal ulceration, which would have the advantage of inducing CFTR dysfunction rapidly in adults, minimizing the contribution of compensatory mechanisms. Furthermore, the use of adult rats as test subjects enables the use of the well-described acute duodenal ulcerogen cysteamine (34, 45).

Verkman’s group recently discovered a thiazolidinone compound, named CFTRinh-172, that has shown considerable promise as an acute and selective inhibitor of CFTR function. In early studies, they demonstrated a lack of toxicity for whole animal studies combined with a high potency and selectivity for CFTR over other ion channels and transporters as well as inhibition of intestinal secretion (33, 51). This high potency and selectivity enabled the use of CFTRinh-172 in rats, as opposed to less potent and more toxic anion channel inhibitors such as NPPB, which are too toxic for whole animal experiments (32). Therefore, to further test our hypothesis that...
diminished DBS due to CFTR dysfunction is associated with protection against acid-related duodenal injury, we examined the effect of CFTRinh-172 on DBS and cysteamine-induced duodenal ulceration in rats.

METHODS

Chemicals and animals. CFTRinh-172 was synthesized according to the published chemical structure of CFTRinh-172 (33) and purified with HPLC, with the structure verified by nuclear magnetic resonance. CFTRinh-172 was dissolved with DMSO at 1 mg/10 µl for intraperitoneal injection and 1 mM for duodenal perfusion; the stock solution was kept at -20°C until use. DMSO (1%) in saline was used as the vehicle for intraperitoneal injection. Cysteamine, ethanolaamine, and other chemicals were obtained from Sigma (St. Louis, MO). Cysteamine or ethanolaamine was dissolved in ethanol and diluted with saline at 50 mg/ml.

All studies were performed with the approval of the Veterans Affairs Institutional Animal Care and Use Committee. Male Sprague-Dawley rats weighing 200–250 g (Harlan; San Diego, CA) were fasted overnight but had free access to water.

Measurement of HCO₃⁻ secretion. Under isoflurane anesthesia (1.5–2.0%) using a rodent anesthesia inhalation system (Summit Medical Systems; Bend, OR), rats were placed supine on a recirculating heating block system (Summit Medical) to maintain body temperature at 36–37°C, as monitored by a rectal thermometer. Pre-warmed saline was infused via the right femoral vein at 1.08 ml/h using a Harvard infusion pump (Harvard Apparatus; Holliston, MA); blood pressure was monitored via a catheter placed in the left femoral artery using a pressure transducer (Kent Scientific; Torrington, CT). DBS was measured with the pH stat method through the duodenal loop as previously described (4, 14). In brief, the stomach and duodenum were exposed, and the forestomach wall was incised using a thermal cautery device (Geiger Medical Technologies; Monarch Beach, CA). A polyethylene tube (5 mm diameter) was inserted through the incision until it was 0.5 cm caudal to the pyloric ring, where it was secured with a nylon ligature. The distal duodenum was ligated proximal to the ligament of Treitz before the duodenal loop was filled with 1 ml saline prewarmed to 37°C. The distal duodenum was then incised, through which another polyethylene tube was inserted and sutured into place. To prevent contamination of the perfusate from bile-pancreatic juice, the pancreaticobiliary duct was ligated just proximal to its insertion into the duodenal wall. The resultant closed proximal duodenal loop (perfused length 2 cm) was perfused with prewarmed saline using a peristaltic pump (Fisher Scientific; Pittsburgh, PA) at 1 ml/min. Input (perfusate) and effluent of the duodenal loop were recirculated through a reservoir in which the perfusate was bubbled with 100% O₂. The pH of the perfusate was kept constant at pH 7.0 with a pH stat (models PHM290 and ABU901, Radiometer Analytical; Lyon, France). Secreted HCO₃⁻ was calculated from the amount of 0.01 N HCl used for titration and expressed as micromoles per minute per centimeter.

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The loop was perfused with saline (pH 7.0) for 30 min, during which DBS gradually declined to a steady state, which we define as micromoles per minute per centimeter. Whether CFTRinh-172 affected gastric acid secretion. Furthermore, gastric acid and HCO₃⁻ secretion rates have not previously been measured in the presence of CFTR dysfunction.

We thus measured gastric acid and HCO₃⁻ secretion in the presence of vehicle or CFTRinh-172 pretreatment 1 h before anesthesia induction using previously described methodology (3, 10). A 5-mm-diameter polyvinyl tube was inserted through a forestomach incision made using a thermal cautery, which was secured with a nylon suture. The effluent tube was inserted through the proximal duodenum and secured at the pyloric ring. The stomach was rinsed with prewarmed saline and perfused at 1 ml/min with a Harvard infusion pump. To prevent the gastric content from refluxing into the esophagus, the lower esophagus was gently ligated with nylon sutures without interfering with the vagi- and paraesophageal vasculature, as previously described (49). Effluent pH and [CO₂] were continuously measured with a modification of previously published methods (10) with pH and
CO₂ electrodes, respectively, placed in series using flow-through cells (Micro Flow-Through pH and CO₂ electrodes, Lazar Research Laboratories; Los Angeles, CA). The effluent was collected every 10 min, with acidity measured by back-titration with 0.1 N NaOH using an Autoburette (Radiometer; Copenhagen, Denmark). Gastric acid output was expressed as milliequivalents per 10 min. Total [CO₂] was calculated from pH and [CO₂] using the Henderson-Hasselbalch formula as previously described (3) and expressed as total CO₂ output (in mmol/min). After a 30-min stabilization, time was set as t = 0. The basal acid secretion was measured for 30 min (t = 0–30 min), followed by a stimulated secretion with a submaximal dose of pentagastrin (16 μg·kg⁻¹·h⁻¹ continuous iv) for 60 min (t = 30–90 min) as previously described (48).

**Statistics.** All data are expressed as means ± SE of 6 rats/group. Comparisons between groups were made by one-way ANOVA, followed by Fischer’s least-significant difference test. Comparisons of two groups were assessed by unpaired, one-tailed t-test. Nonparametric injury data were analyzed using the Mann-Whitney test. P values of 0.05 were taken as significant.

**RESULTS**

**Effects of CFTRinh-172 perfusion on basal and acid-induced DBS.** Because the CFTR is present in the apical membrane of duodenal epithelial cells (9), we examined the effect of luminal application of CFTRinh-172 on basal and acid-induced DBS. CFTRinh-172 (0.1–10 μM) did not affect basal DBS (Fig. 1A). DBS was increased after acid exposure, whereas luminal perfusion with 10 μM CFTRinh-172 (dissolved in pH 2.2 saline) abolished acid-induced increases of DBS (Fig. 1B), indicating that luminal CFTRinh-172 inhibited acid-induced but not basal DBS, suggesting that its effect is limited to acid-activated CFTR function.

**Effects of CFTRinh-172 pretreatment on basal and acid-induced DBS.** Acid-induced DBS was abolished 1 or 3 h after CFTRinh-172 treatment, whereas basal DBS was unaltered (Fig. 2A), confirming the acute inhibitory effect of CFTRinh-172 on DBS, similar to the effect of the compound on intestinal anion secretion (51). Basal DBS was increased 24 h after CFTRinh-172 treatment, whereas acid-induced DBS was still reduced (Fig. 2B), indicating that the inhibition of acid-induced DBS with CFTRinh-172 lasted at least 24 h.

**Effects of CFTRinh-172 pretreatment on cysteamine-induced duodenal ulceration.** Duodenal ulceration was observed 24 h after treatment with a ulcerogenic dose of cysteamine (300 mg/kg; Fig. 3A), which was not observed with a nonulcerogenic dose of cysteamine (300 mg/kg; Fig. 3B).
genic dose of cysteamine (100 mg/kg) or ethanolamine treatment (data not shown). Two of the six ulcers were kissing ulcers. Pretreatment with CFTRinh-172 1 h before cysteamine treatment reduced duodenal ulcer formation, as assessed at 24 h after cysteamine treatment (Fig. 3B). There were no kissing or multiple ulcers observed in the CFTRinh-172-pretreated group. This macroscopic observation was confirmed in the histological H&E-stained sections, where transmural ulceration with massive necrosis was observed in the duodena of cysteamine-treated rats (Fig. 3C). Occasional mucosal erosions, but no deep ulcers, were observed in the duodena of CFTRinh-172-pretreated rats (Fig. 3D). As shown in Fig. 3D, for example, no erosions were observed, but there were a few sloughed epithelial cells, subepithelial edema in some villi, and minimal hemorrhage in the villous mucosa (lamina propria mucosa) and in the submucosa. The ulcer area was significantly attenuated by CFTRinh-172 pretreatment 1 h before cysteamine treatment (Fig. 3E). The ulcer score was also reduced by CFTRinh-172 pretreatment (Fig. 3F).

**DISCUSSION**

We demonstrated that CFTRinh-172 acutely inhibited stimulated duodenal CFTR function, as measured as acid-induced DBS, and attenuated cysteamine-induced duodenal ulceration. Furthermore, regardless of the effect of CFTRinh-172 on gastric acid and DBS, CFTR inhibition protected the duodenal mucosa from cysteamine-induced acid-related injury, supporting our hypothesis that DBS and injury do not always correlate. These studies further suggest that CFTR dysfunction, with a consequent impairment of stimulated DBS, does not increase the susceptibility of the duodenal mucosa to acid-induced injury.

**Effects of CFTRinh-172 pretreatment on gastric acid and HCO\(_3\)\(^{-}\) secretion.** CFTRinh-172 pretreatment had no effect on either basal or pentagastrin-induced acid secretion in the stomach (Fig. 4A). Furthermore, there was no difference in total gastric CO\(_2\) output between vehicle- or CFTRinh-172-treated groups (Fig. 4B), suggesting that the CFTR does not directly mediate gastric acid or HCO\(_3\)\(^{-}\) secretion.
Our study is the first to examine the effect of CFTRinh-172 on DBS. CFTR inhibition with a single intraperitoneal dose of CFTRinh-172 was observed at least 1 h after its administration and lasted at least 24 h thereafter, consistent with earlier studies by Verkman’s group, although they showed the inhibition only up to 6 h in a enterotoxin-induced intestinal diarrhea model (33, 44, 51). Verkman’s group has also shown that CFTRinh-172 has measurable concentrations in the small intestine 60 min after an intravenous injection (44). The inhibitor did not affect basal DBS but rather only inhibited augmented DBS in response to luminal acid challenge. This result is discordant with those observed in vivo in murine models of genetic CFTR dysfunction and in biopsies obtained from patients with CF (8, 18, 19, 39). This discrepancy may reflect the induction of acute rather than chronic CF dysfunction. Also likely is that CFTRinh-172 only inhibited activated CFTR, because CFTRinh-172 only inhibits activated and not basal CFTR function in a variety of tissues and species (33, 43, 51, 52). CFTRinh-172 most likely inhibits CFTR by binding to the cytoplasmic loops of the cAMP-activated protein (33, 43, 44). This would explain our observation that systemically applied CFTRinh-172 diminishes the acid-induced DBS increase, suggesting that its cytoplasmic binding site is related to CFTR activation, such as by protein kinase phosphorylation (21).

Our interest in CF in the context of duodenal injury originated in the clinical observation that CF patients, despite low DBS and rates of high acid secretion, have an unexpectedly low prevalence of duodenal ulceration (6, 15, 29, 31, 40), which we term the “CF paradox” (24). In our studies of DBS, we used genetic and pharmacological models of CFTR dysfunction to study duodenal defense mechanisms. We inhibited CFTR function with nonselective anion inhibitors such as NPPB (4) and measured HCO₃⁻ secretion and mucosal injury in CFTR mutant mice (17). Nevertheless, the acid-induced injury we previously assessed was early and superficial, as detected by propidium iodide, and not likely fully comparable to deep injury expressed clinically as ulceration. Furthermore, genetic CFTR mutations may be compensated during development, perhaps confounding the effect of CFTR dysfunction.

The most accepted rodent duodenal ulcer model is the cysteamine rat model, which has the advantages of rapid and reproducible formation of duodenal ulcers by a mechanism thought to involve a diminution of duodenal host defenses to luminal acid, such as DBS, mucus secretion, and mucosal blood flow, and enhancement of aggressive factors such as acid and pepsin secretion (20, 27, 50). Other hypotheses regarding the mechanism of action of cysteamine include somatostatin depletion (47) and alteration of the mucosal redox state (25). Despite the increased acid secretion observed in cysteamine-treated rats, similar duodenal ulceration was not observed in other models of acid hypersecretion, such as pentagastrin-treated rats, strongly suggesting that a decrease of mucosal protective factors underlies the primary ulcerogenic effect of cysteamine treatment (27, 30, 35). Bridén et al. (7) have suggested that cysteamine impairs the augmentation of HCO₃⁻ secretion in response to luminal acid while not affecting prostaglandin-stimulated DBS, consistent with an impairment of duodenal acid-sensing mechanisms. Cysteamine rapidly induces duodenal epithelial injury, with changes detectable 30 min after administration (50), accompanied by increased gastric acid output (27) and decreased duodenal blood flow (1, 50), with ulcers appearing within 24 h (30, 45). The time course of cysteamine-induced ulcer induction fortuitously matched well with the duration of inhibition of DBS by CFTRinh-172. Interestingly, enhanced basal DBS was observed 24 h after CFTRinh-172 treatment, suggesting that CFTR inhibition may upregulate the basal HCO₃⁻ secretory pathway, which appears to be mediated by a mechanism distinct from the acid-stimulated pathway (22). We speculate that CFTRinh-172 impairs cysteamine-induced ulceration by limiting the HCO₃⁻ exit from epithelial cells, increasing the overall cellular buffer capacity and thus limiting cellular acidification during luminal acid stress.

Whether CFTR inhibition affects any defence mechanism other than DBS, such as blood flow or mucus secretion, is still unknown. Histological sections revealed that cysteamine-induced ulceration was inhibited with CFTRinh-172 pretreatment, whereas minimum injury such as epithelial cell sloughing, subepithelial edema, and mild duodenitis was present, suggesting that the protective effects of CFTRinh-172 mostly affected epithelial cells, with lesser effects on the subepithelial mucosa. Because cysteamine increases gastric acid output and decreases DBS and Brunner’s gland secretions (7, 16, 26, 38), CFTRinh-172 might selectively protect epithelial cells from luminal injurious factors while having lesser effects on submucosal factors such as decreased blood flow (50), reducing injury depth.

In conclusion, CFTRinh-172 is a useful tool to produce acute CFTR dysfunction in rodents as a CF model and also to examine the role of CFTR in models of up to 24-h duration.
CFTR INHIBITION AND DUODENAL DEFENSE

CFTR inhibition reduces acid-induced DBS but attenuates duodenal ulcer formation, supporting our hypothesis that CFTR dysfunction is associated with protection of the duodenal mucosa from acid-related injury.

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