NHE3 inhibition activates duodenal bicarbonate secretion in the rat

Osamu Furukawa,2,5 Luke C. Bi,4 Paul H. Guth,1 Eli Engel,3 Masahiko Hirokawa,2,4 and Jonathan D. Kaunitz1,2,5
1Greater Los Angeles Veterans Affairs Healthcare System, 2Department of Medicine, School of Medicine, and 3Department of Biomathematics, University of California Los Angeles, 4San Fernando Valley Internal Residency Program, and 5CURE: Digestive Diseases Research Center, Los Angeles, California 90073

Submitted 21 February 2003; accepted in final form 18 July 2003

Furukawa, Osamu, Luke C. Bi, Paul H. Guth, Eli Engel, Masahiko Hirokawa, and Jonathan D. Kaunitz. NHE3 inhibition activates duodenal bicarbonate secretion in the rat. Am J Physiol Gastrointest Liver Physiol 286: G102–G109, 2004.—We examined the effect of inhibition of Na+/H+ exchange (NHE) on duodenal bicarbonate secretion (DBS) in rats to further understand DBS regulation. DBS was measured by using the pH-stat method and by using CO2-sensitive electrodes. 5-([N,N-dimethyl]-amiloride (50 μM; DMA), a concentration that selectively inhibits the NHE isoforms NHE1 and NHE2, but not NHE3, did not affect DBS. Nevertheless, 3 mM DMA, a higher concentration that inhibits NHE1, NHE2, and NHE3, significantly increased DBS. Moreover, S1611 and S3226, both specific inhibitors of NHE3 only, or perfusion with Na+-free solutions, dose dependently increased DBS, as measured by pH-stat and CO2-sensitive electrode, without affecting intracellular pH. Coperfusion with 0.1 μM indomethacin, 0.5 mM DIDS, or 1 mM methazolamide did not affect S3226-inhibited DBS. Nevertheless, coperfusion with 0.1 and 0.3 mM 5-nitro-2-(3-phenylpropylamino) benzoic acid, which inhibits the cystic fibrosis transmembrane conductor regulator (CFTR), dose dependently inhibited S3226-inhibited DBS. In conclusion, only specific apical NHE3 inhibition increased DBS, whereas prostaglandin synthesis, Na+-HCO3 cotransporter activation, or intracellular HCO3 formation by carbonic anhydrase was not involved. Because NHE3 inhibition-increased DBS was inhibited by an anion channel inhibitor and because reciprocal CFTR regulation has been previously shown between NHE3 and apical membrane anion transporters, we speculate that NHE3 inhibition increased DBS by altering anion transporter function.

epithelial cells; cystic fibrosis transmembrane conductance regulator; back titration; S3226

DUODENAL EPITHELIAL BICARBONATE secretion (DBS) is one of the most important mechanisms by which the duodenum is protected from the injurious effects of secreted gastric acid (6, 16). DBS is regulated by humoral factors such as PGE2, VIP, glucagon, gastric inhibitory peptide, and the enteric nervous system (6). These factors promote cAMP production, which stimulates the cystic fibrosis transmembrane conductor regulator (CFTR), an apical anion channel (19, 20), and the basolateral Na+-HCO3 cotransporter (NBC1), an HCO3 uptake pathway (4, 22, 35). Recently, six Na+/H+ exchanger (NHE) isoforms have been cloned. Of these isoforms, NHE1, 2, and 3 are expressed in the intestine of humans, rabbits, and rats (10, 21). In the small intestine, particularly in the duodenum, apical NHE2 and NHE3 are expressed in human, rabbits, rats, and mice (10, 21, 34). In the colonic surface mucosa, an apical NHE3 plays a key absorptive role for Na+, concomitant with H+ excretion (31). In contrast, NHE2 may promote Na+ absorption from colonic crypts (12), whereas playing only a minor role in overall small intestinal electrolyte transport (18, 29).

In a recently published clinical study (36), inhibition of NHE2 and NHE3 by amiloride increased DBS. This increased DBS was thought to result from decreased NHE2- and NHE3-mediated H+ secretion into the lumen, increasing the amount of measured titratable alkalinity. Although it is plausible that an apparent rather than a true increase of DBS was measured, the constraints imposed by clinical studies prevented differentiation of these two possibilities. On the basis of these data, we thus formulated two hypotheses: 1) that the increase of titratable alkalinity observed during previously observed amiloride perfusion was, in part, reflective of a true increase of DBS; and 2) that the increased DBS resulted from NHE3 inhibition. To test these hypotheses, we examined the effect of the relatively nonselective NHE inhibitor, 5-(N, N-dimethyl)-amiloride (DMA), and the more selective NHE3 inhibitors, S1611 and S3226, on DBS, as measured by the CO2-sensitive electrode and pH-stat method in rats.

MATERIALS AND METHODS

Animals and Chemicals

Male Sprague-Dawley rats weighing 225–275 g (Harlan Laboratories, San Diego, CA) were fasted overnight but allowed free access to tap water. All studies were approved by the Animal Use Committee of the Greater Los Angeles Veterans Administration Healthcare System.

DMSO, DIDS, N-methyl-d-glucamine (NMDG), 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), methazolamide, indomethacin, HEPES, and other chemicals were obtained from Sigma (St. Louis, MO). S1611 and S3226 (37, 42, 44) were a kind gift of Aventis Pharma Deutschland (Frankfurt am Main, Germany). PGE2 was obtained from Oxford Biochemical (Oxford, MS). HEPES-saline solution contained 135 mM NaCl and 20 mM HEPES at pH 7.0. S1611, S3226, DMA, NPPB, methazolamide, and indomethacin were dissolved with DMSO, and DIDS was dissolved with distilled water to make concentrated stock solutions.

Measurement of Duodenal HCO3 Secretion

Preparation of duodenal loop. Duodenal loops were prepared and perfused to measure duodenal HCO3 secretion as described previously (4). Briefly, rats were anesthetized with urethane (1.25 g/kg ip).

http://www.ajpgi.org
the abdomen was incised, and both stomach and duodenum were exposed. A duodenal loop (2 cm) was made distal to the pyloric ring. To prevent contamination of the perfusate from bile-pancreatic juice, the pancreaticobiliary duct was ligated just proximal to its insertion into the duodenal wall.

**pH-stat method.** The resultant closed proximal duodenal loop was perfused with prewarmed saline by using a peristaltic pump at 1 ml/min. Input and effluent of duodenal loop were circulated through a reservoir, in which the perfusate was bubbled with 100% O2 gas (3, 4). The pH of the perfusate was kept at pH 7.0 with a pH-stat (models PHM290 and ABU901; Radiometer Analytical, Lyon, France). For back titration, the amount of 10 mM HCl added to keep the pH of the perfusate before the addition of S3226.

**CO2 measurements.** Total dissolved CO2 was measured by the CO2 electrode gas sensing electrode (model 950200; Thermo Orion, MA) connected to a pH meter (model PHM 62; Radiometer, Copenhagen, Denmark) (3, 4). Duodenal loops were prepared and perfused with 20 mM HEPES containing saline (pH 7.0) at a rate of 1 ml/min as described above, with effluent collected every 5 min. We then added 0.5 ml of a 1 M citrate buffer (pH 4.5) to the sample (5 ml) to convert free HCO3 to CO2, followed by measurement of electrode potential with the CO2 electrode. Total dissolved CO2 concentration ([CO2]t) was calculated according to a calibration curve by using freshly prepared 0.1, 1, and 10 mM Na+/HCO3 solutions as standards, which generate 0.1, 1, and 10 mM [CO2], respectively (3). After reaching stability for at least 15 min as well as the pH-stat method, S1611, S3226, or DMA was added to the perfusate.

**Image analysis**

Fluorescence of the microscopically observed chambered segment of duodenal mucosa at 515 nm emission was recorded with a cooled charge-coupled device video camera (Hamamatsu Orca-EN; Hamamatsu, Bridgewater, NJ). Fluorescence intensity of the selected area was measured by first capturing the image by using an Apple G4 microcomputer and digitized with area of interest defined, and intensity was measured by using image analyzer software (OpenLab; Improvision, Lexington, MA). The intensity of emitted fluorescence at 495 nm stimulation is pH dependent, whereas that at 450 nm is not. Therefore, 450 and 495 nm filters, narrow band-pass interference filters (Chroma, Brattleboro, VT) were used and each image was captured every 5 min. Readings were taken at 10 s before and after each time point. The paired readings needed to calculate a fluorescence ratio were thus taken at a maximum of 20 s apart. Image analysis was performed on the recorded images as follows: initially three small areas of a duodenal epithelium were selected at random and then followed throughout the experiment. In vitro calibration and background compensation using an aqueous solution containing 0.2 μM BCECF free acid were done as described previously (5, 24).

**Statistics**

Comparisons between groups were made by one-way ANOVA followed by Fisher’s least significant difference test. *P < 0.05* was taken as significant.

![Image](https://www.ajpgi.org)
RESULTS

Effect of DMA on DBS

Initial experiments were conducted by using the concentration-dependent NHE inhibitor DMA, to selectively inhibit NHE isoforms. Basal DBS, as measured with the CO\textsubscript{2}-sensitive electrode, was 0.08–0.10 µmol\textperiodcentered min\textsuperscript{-1}cm\textsuperscript{-1}. NHE2 activity was inhibited with 50 µM DMA perfused into the duodenal loop. As seen in Fig. 1, DBS was unchanged for at least for 1 h. In contrast, 3 mM DMA gradually increased DBS to ~0.15 µmol\textperiodcentered min\textsuperscript{-1}cm\textsuperscript{-1} 30 min after the addition, with the increased secretion lasting for 60 min.

Effects of S1611 and S3226 on DBS

To further confirm the role of NHE3 inhibition on DBS, we examined the effect of the more selective NHE3 inhibitors S1611 and S3226. Similar to the effects of 3 mM DMA, the addition of 1 or 10 µM of S3226 to the perfusate, which selectively inhibits NHE3 (37, 42), gradually and dose dependently increased DBS, as measured by the CO\textsubscript{2}-sensitive electrode method. In particular, 10 µM S3226 significantly stimulated DBS within 10 min after the addition, reaching a peak of 1.5 times basal (Fig. 2A). After withdrawal of S3226, DBS remained elevated for 40 min and was further stimulated by the

---

Fig. 2. Effects of S3226 on DBS measured by the CO\textsubscript{2}-sensitive electrode method. A: perfusion with S3226 elevated DBS. B: withdrawal of S3226 did not affect DBS over the 40-min monitoring period, but injection of PGE\textsubscript{2} further raised secretion, indicating that S3226 did not affect the ability of the mucosa to secrete. C: when measured by the pH-stat method, results are qualitatively similar to secretion measured by CO\textsubscript{2} electrode.

AJP-Gastrointest Liver Physiol • VOL 286 • JANUARY 2004 • www.ajpgi.org
addition of PGE$_2$ (0.1 mg/kg iv; Fig. 2B). The effects of 1 or 10 μM S3226 on DBS were confirmed by using the pH-stat method. Basal DBS measured by the pH-stat method was ~0.05 μmol·min$^{-1}$·cm$^{-1}$. The addition of 1 or 10 μM S3226 to the circulating perfusate gradually and dose dependently increased DBS, reaching a peak of 1.5 times basal with 10 μM S3226 (Fig. 2C). We then examined the effect of S1611, which has a median inhibitory concentration (IC$_{50}$) for rat NHE3 greater than that of S3226 (0.69 vs. 0.23 μM) (44). Perfusion with 10 μM S1611 produced similar but less marked DBS stimulation than that of S3226, as measured by the CO$_2$-sensitive electrode method (Fig. 3).

**Effects of NMDG on DBS**

We then examined the role of perfusate Na substitution on DBS. Removal of Na from the perfusate inhibits NHE3 function by decreasing the Na available for exchange (34). As seen in Fig. 4, substitution of NMDG for Na in the perfusate rapidly increased DBS, as measured by the CO$_2$-sensitive electrode method, reaching a peak of 1.6 times basal within 15–20 min after initial perfusion with subsequent stabilization at a higher level.

**Effects of Indomethacin, Methazolamide, DIDS, and NPPB on DBS**

To further elucidate the mechanism by which inhibition of NHE3 increased DBS in rats, we examined the effects of several compounds on S3226-stimulated DBS, measured by using the CO$_2$-sensitive electrode method. Indomethacin (0.1 μM), which completely inhibits acid-induced DBS but did not affect PGE$_2$-stimulated DBS in rats (17), did not affect basal or S3226-induced DBS (Fig. 5). We then examined the effect of methazolamide, a permeant carbonic anhydrase inhibitor, on DBS. Methazolamide (1 mM) slightly decreased basal DBS from ~0.1 to ~0.08 μmol·min$^{-1}$·cm$^{-1}$ within 10–15 min after addition (Fig. 6A). This decreased DBS remained unchanged for 1 h. In the presence of methazolamide, 10 μM of S3226 increased DBS to a maximum value of 0.14 μmol·min$^{-1}$·cm$^{-1}$, somewhat less than the maximum value observed S3226 alone. When Δ increases (the area under the curve 60 min after S3226 addition, relative to the baseline recorded prior S3226 addition) were calculated, no significant difference between DBS after the addition of S3226 alone and methazolamide plus S3226 was observed (Fig. 6B). To examine the role of NBC1 on S3226-stimulated DBS, we tested the effect of 0.5 mM DIDS, which inhibits DBS presumably by inhibition of cellular HCO$_3$ uptake (3, 4). DIDS (0.5 mM)

---

**Fig. 3.** Effects of S1611 on DBS measured by the CO$_2$-sensitive electrode method. Results are qualitatively similar to those obtained with S3226.

**Fig. 4.** Effect of substitution of perfusate Na with N-methyl-D-glucamine on DBS. Results are qualitatively similar to those obtained with S3226.

**Fig. 5.** Effects of 0.1 μM indomethacin on S3226-augmented DBS measured by the CO$_2$-sensitive electrode method. Indomethacin was added to the perfusate, and then 30 min later S3226 was added to the perfusate.
slightly increased basal DBS within 5–10 min after addition, after which DBS was unchanged. The subsequent addition of 10 μM S3226 increased DBS to a level not different from that observed with S3226 alone (Fig. 7A). DBS (Δ over baseline) for S3226 alone and DIDS plus S3226 were 0.51 ± 0.10 and 0.41 ± 0.14 μmol·60 min⁻¹·cm⁻¹, respectively, with no significant difference between the two groups (Fig. 7B). Lastly, to examine the role of the apical anion channel function on DBS, we examined the effect of NPPB on S3226-induced DBS. The addition of 0.1 or 0.3 mM NPPB did not affect basal DBS within 30 min after addition. Nevertheless, 0.1 mM NPPB significantly inhibited S3226-induced DBS 15–50 min after addition. Moreover, 0.3 mM NPPB almost completely inhibited S3226-induced DBS when both inhibitors were included in the perfusate (Fig. 8). Percent inhibitions, as calculated from Δ increases for 0.1 and 0.3 mM NPPB, were 49 and 78%, respectively.

**Effect of S3226 on pHᵢ**

In the last series of studies, we examined the effect of S3226 on pHᵢ to determine whether S3226 decreased pHᵢ as a signal for DBS. Because NHE1 is a major regulator of pHᵢ and NHE3 might also be involved in pHᵢ regulation in duodenal epithelial cells (34), we hypothesized that NHE3 inhibition might decrease pHᵢ, serving as a signal for subsequent DBS. Our prior studies (4, 5) revealed that other stimulants of DBS, such as acid perfusion, lowered pHᵢ before the onset of DBS. As seen in Fig. 9, 10 μM S3226 had no effect on duodenal epithelial cells perfused in situ.

---

Fig. 6. Effects of 1 mM methazolamide on S3226-augmented DBS measured by the CO₂-sensitive electrode method. A: methazolamide was added to the perfusate, and then 1 h later S3226 was added to the perfusate. B: change in HCO₃⁻ output for 1 h after the addition of S3226.

Fig. 7. Effects of 0.5 mM DIDS on S3226-augmented DBS measured by the CO₂-sensitive electrode method. A: DIDS was added to the perfusate, and then 30 min later, S3226 was added to the perfusate. B: change in HCO₃⁻ output for 1 h after S3226 addition.
DISCUSSION

We used low (50 μM) and high (3 mM) doses of DMA, which inhibited NHE1 and 2 and NHE1–3, respectively, and found that only 3 mM DMA increased DBS, suggesting inhibition of NHE3 is essential for increased DBS. Furthermore, we could confirm that the selective inhibitors of NHE3, S1611, and S3226, as well as Na$^+$-free perfusion, also increased DBS dose dependently by using the CO$_2$-sensitive electrode and the pH-stat methods. These results are consistent with our hypothesis that inhibition of only NHE3 activity but not NHE2 activity increased DBS. These results are consistent with data obtained in transgenic mice that indicated that NHE3 is the primary apical cation exchanger of the small intestine (18, 31). Furthermore, measurements made with CO$_2$-sensitive electrodes confirmed that inhibition of NHE3 increased true DBS and not an apparent increase of DBS due to decreased NHE3-mediated duodenal acid secretion. With the pH-stat method, CO$_2$ produced from secreted H$^+$ combining with secreted HCO$_3^-$ is expelled by bubbling with 100% O$_2$. The pH-stat method thus detects only H$^+$ loss or HCO$_3^-$ increase. Conversely, the CO$_2$-sensitive electrode, only measures HCO$_3^-$ or CO$_2$ concentrations, which are not confounded by epithelial H$^+$ secretion (3, 15).

The mechanism by which inhibition of NHE3 activity increased DBS, especially by S3226, is not well understood. The prostaglandin-cAMP pathway is important for basal and acid-stimulated DBS (16, 41). Indomethacin, generally used as a nonselective cyclooxygenase inhibitor, inhibits basal and acid-induced DBS (41); leukotriene C4/D4 antagonist L-649–923-induced DBS (26); and YM-14673, a thyrotropin-releasing hormone analog induced DBS (40). Nevertheless, because indomethacin did not affect S3226-induced DBS, prostaglan-
NHE3 INHIBITION AUGMENTS DUODENAL BICARBONATE SECRETION

awaits more detailed knowledge regarding the mechanism of NHE3 inhibition by S3226 and S1611.

CFTR plays a crucial role for HCO₃⁻ secretion. NPPB inhibits anion channels, including CFTR, inhibiting HCO₃⁻ secretion in vitro Ussing chamber studies in mice (14). Furthermore, in CFTR knockout mice, basal DBS is reduced by ~80%, as is PGE₂- and VIP-stimulated DBS (19, 20). It is of interest that in recent studies, the COOH-terminal postynaptic density protein-95/synapse-associated protein-90/Disclarge/zenula occulden-1 (PDZ) domain of CFTR associates with NHE3 (30) and with other molecules important in the regulation of anion secretion such as CFTR and the apical anion exchanger downregulated in adenoma (DRA; SLC26A3), or other members of the SLC26A family that serve as intestinal epithelial apical anion exchangers (28, 43). The PDZ binding motif of CFTR and NHE3 are both thought to bind NHE regulatory factor (27). Not only is there evidence for a molecular association between CFTR and NHE3, but also there is a suggestion that CFTR inversely regulates NHE3 activity. The cyclic nucleotide cAMP increases CFTR-mediated Cl⁻ secretion while inhibiting NHE3-mediated Na absorption (45). Stable NHE3 expression downregulates CFTR activity in cultured renal cells (9). In NHE3 null mouse colon, DRA transcripts, which are associated with HCO₃⁻ secretion, are in increased abundance (32). Furthermore, in CFTR knockout mouse intestine, or in pancreatic-derivd PS120 cells transfected with a PDZ-deficient CFTR transcript, the ability of cAMP to inhibit NHE3 activity is impaired (1, 13). Thus there is a plausible molecular mechanism underlying the reciprocal CFTR/anion transporter interaction, although no group before us has demonstrated the upregulation of CFTR function associated with acute inhibition of NHE3 activity, particularly in an in situ preparation.

Taken together, inhibition of apical membrane NHE3 activity by S3226, S1611, perfusate Na removal, and DMA increased DBS. Because the CO₂ concentration increased in parallel with titratable alkalinity, NHE3 inhibition increased HCO₃⁻ secretion in addition to decreasing luminal H⁺ entry. Prostaglandin synthesis, Na⁺/HCO₃⁻ cotransporter activation, intracellular acidification, or intracellular HCO₃⁻ formation by carbonic anhydrase were not involved in this effect. Because NHE3 and HCO₃⁻ secretion are inversely regulated, we speculate that NHE3 inhibition upregulated CFTR or DRA function via protein-protein or protein-DNA interactions but did not affect other pathways involved with DBS.

ACKNOWLEDGMENTS

The authors thank Dipty Shah and Ritu Jain for technical assistance and Rebecca Cho for artistic and secretarial support.

DISCLOSURES

This study was supported by Veterans Affairs Merit Funds and National Institute of Diabetes and Digestive and Kidney Diseases Grant 2-R01-DK54221.

REFERENCES


22. Janecki AJ, Montrose MH, Zinniak P, Zewebaum A, Tse CM, Khurana S, and Donowitz M. Subcellular redistribution is involved in acute regulation of the brush border Na⁺/H⁺ exchanger isoform 3 in...