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Research Article

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Endogenous Endothelins Mediate Increased Distal Tubule Acidification Induced by Dietary Acid in Rats

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Abstract

We examined if endogenous endothelins mediate the decreased HCO_3^- secretion and increased H^+ secretion in vivo-perfused distal tubules of rats fed dietary acid as $(\text{NH}_4)_2\text{SO}_4$. Animals given $(\text{NH}_4)_2\text{SO}_4$ drinking solution had higher endothelin-1 addition to renal interstitial fluid than those given distilled H_2O (480 ± 51 vs. 293 ± 32 fmol g kidney $\text{wt}^{-1} \text{min}^{-1}$, respectively, $P < 0.03$). $(\text{NH}_4)_2\text{SO}_4$ -ingesting animals infused with bosentan (10 mg/kg) to inhibit A- and B-type endothelin receptors had higher HCO_3^- secretion than baseline $(\text{NH}_4)_2\text{SO}_4$ animals (-4.7 ± 0.4 vs. -2.4 ± 0.3 pmol $\text{mm}^{-1} \text{min}^{-1}$, $P < 0.01$), but $(\text{NH}_4)_2\text{SO}_4$ animals given a specific inhibitor of A-type endothelin receptors (BQ-123) did not (-2.0 ± 0.2 pmol $\text{mm}^{-1} \text{min}^{-1}$, $P = \text{NS}$ vs. baseline). H^+ secretion was lower in bosentan-infused compared with baseline $(\text{NH}_4)_2\text{SO}_4$ animals (27.7 ± 2.5 vs. 43.9 ± 4.0 pmol $\text{mm}^{-1} \text{min}^{-1}$, $P < 0.03$), but that for BQ-123-infused $(\text{NH}_4)_2\text{SO}_4$ animals was not (42.9 ± 4.2 pmol $\text{mm}^{-1} \text{min}^{-1}$, $P = \text{NS}$ vs. baseline). Bosentan had no effect on distal tubule HCO_3^- or H^+ secretion in control animals. The data show that dietary acid increases endothelin-1 addition to renal interstitial fluid and that inhibition of B- but not A-type endothelin receptors blunts the decreased HCO_3^- secretion and increased H^+ secretion in the distal tubule of animals given dietary acid. The data are consistent with endogenous endothelins as mediators of increased distal tubule acidification induced by dietary acid. (*J. Clin. Invest.* 1997; 99: 2203–2211.) Key words: bicarbonate • bosentan • interstitium • micropuncture • proton • secretion

Introduction

Dietary acid increases acidification in the distal tubule in vivo (1–3), but the factors that induce this response are unknown. This dietary maneuver might increase distal tubule acidification directly through changes in acid–base parameters of body fluids and/or indirectly by altering production of substances that modulate distal tubule acidification. In previous studies from this laboratory, animals ingesting the acid-producing salt $(\text{NH}_4)_2\text{SO}_4$ increased distal tubule acidification and urine acid

excretion without sustained changes in plasma acid–base parameters (3). Furthermore, intracellular pH of cultured renal epithelial cells chronically (48 h) exposed to acid media was not different from control, yet these cells had increased Na^+/H^+ antiporter activity (4). These data suggest that persistent alterations in plasma or intracellular fluid acid–base parameters are not necessary to permit a sustained increase in renal epithelial acidification. Thus, dietary acid might increase distal nephron acidification indirectly by altering production of substances that more directly influence the components of distal tubule acidification. The mechanism by which the distal tubule increases acidification in response to dietary acid might suggest substances responsible for inducing the change. Our previous studies showed that dietary acid decreases HCO_3^- secretion and increases H^+ -secreting capacity in distal tubules in vivo (3). Endothelin-1 (ET-1),¹ an agent made by collecting tubules (5) and renal microvascular endothelium (6), decreases distal tubule HCO_3^- secretion induced by dietary HCO_3^- (7) and increases Na^+/H^+ exchanger (NHE)-3 activity in cultured renal epithelia (8). The data show that directional changes in components of distal tubule acidification induced by dietary acid are consistent with ET-1 actions on tubule $\text{HCO}_3^-/\text{H}^+$ secretion.

The present studies used free-flow and in vivo micropuncture to test the hypothesis that augmented endothelin secretion mediates increased distal tubule acidification induced by dietary acid. The data show that dietary acid increases ET-1 addition to renal interstitial fluid, a fluid with access to both the renal epithelium and endothelium. Furthermore, the studies show that endothelin receptor inhibition blunts both the decrease in distal tubule HCO_3^- secretion and increase in H^+ -secreting capacity induced by dietary acid. The data support the hypothesis that endothelin mediates the increase in distal tubule acidification induced by dietary acid.

Methods

Male and female Munich-Wistar rats (Harlan Sprague-Dawley Co., Houston, TX) weighing 240–271 g were used. Previous studies showed that dietary $(\text{NH}_4)_2\text{SO}_4$ decreased HCO_3^- secretion and increased H^+ secreting capacity in the rat distal tubule in vivo (3). We used the same $(\text{NH}_4)_2\text{SO}_4$ -ingesting protocol in the present studies and these animals will be referred to as acid ingesting. Experimental and control animals eating a minimum electrolyte diet (ICN Nutritional Biochemicals, Cleveland, OH) received 40 mM $(\text{NH}_4)_2\text{SO}_4$ drinking solution and distilled H_2O , respectively, for 7–10 d before micropuncture. Because $(\text{NH}_4)_2\text{SO}_4$ -ingesting animals had higher urine flow than control and because diuresis might increase urine ET-1 excretion (9), we studied a second control group ingesting 40 mM Na_2SO_4 drinking solution that had similar drinking volumes and urine flows to $(\text{NH}_4)_2\text{SO}_4$ animals. Bosentan (Hoffman-LaRoche, Basel,

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1. Abbreviations used in this paper: ET-1, endothelin-1; J_v , fluid reabsorption; NAE, net acid excretion; NHE, Na^+/H^+ exchanger; RIF, renal interstitial fluid.

Switzerland), a nonpeptide endothelin_A and endothelin_B receptor antagonist (10), was infused (10 mg/kg i.v.) to antagonize actions of endogenous endothelins. This bosentan dose inhibits initial depressor and sustained pressor responses to ET-1 > 60% as long as 6 h (10). To distinguish endothelin_A- from endothelin_B-mediated effects, we infused a comparison animal group with BQ-123 (Bachem California, Torrance, CA), a selective endothelin_A receptor antagonist (11), 1 mg/kg i.v. followed by 0.1 mg/kg per h. This BQ-123 dose inhibited pressor responses to both stimulated endogenous endothelins and infused ET-1 (12).

Urine net acid excretion. Daily net acid excretion (NAE) was measured as described (13) in four each of control, (NH₄)₂SO₄, and Na₂SO₄ animals fed drinking solution and diet as described. Because rats of similar weight ingested 15.6±0.6 g/d of diet when drinking distilled H₂O, 16.7±0.8 g/d for the (NH₄)₂SO₄ solution, and 15.9±0.6 g/d for the Na₂SO₄ solution, each animal was fed exactly 15 g/d of diet to ensure that each group ingested the same amount. The diet contained 20% protein and the following electrolytes (in μeq/g diet): 21.7 Na⁺, 43.5 K⁺, and 13.8 Cl⁻. All groups drank distilled H₂O with diet for 48 h, and then were given either 40 mM (NH₄)₂SO₄, 40 mM Na₂SO₄, or distilled H₂O (control) for an additional 7 d while in metabolic cages. On each of the subsequent 7 d, animals were anesthetized with ketamine HCl (100 mg/kg body wt; Parke-Davis, Morris Plains, NJ) and bladder urine anaerobically obtained by percutaneous puncture with a 25-gauge needle as done previously (13) for NAE measurement. After anesthesia recovery and excreting blood-free urine, they returned to metabolic cages to continue their drinking solutions. We examined NAE in an additional set of bosentan-infused compared with vehicle-infused animals from each of the three experimental groups after they had ingested their drinking solutions and diet for 7 d. On the evening of the 7th day, animals were anesthetized as described and 10 mg/kg bosentan or vehicle infused into the jugular vein. The wound was closed with surgical clips and NAE was measured as described for the next 12 h.

Microdialysis technique for measurement of renal interstitial fluid ET-1. Renal interstitial fluid (RIF) ET-1 addition was estimated using microdialysis of the renal cortical interstitium (14). A microdialysis apparatus was constructed from a 5-mm-long piece of hollow fiber dialysis tubing (molecular mass cutoff 5,000 D; Hospal, Meyzieu, France) with 0.1-mm inner diameter as described (14). Each end of the dialysis tubing was connected to a 25-cm-long polyethylene tube (0.12-mm inner diameter, 0.65-mm outer diameter; Bioanalytical systems, Indianapolis, IN) and sealed in place with cyanoacrylic glue (14). The left kidney was exposed through a flank incision in anesthetized rats and its renal capsule penetrated with a 31-gauge needle that was tunneled in the outer renal cortex ~ 1 mm from the renal surface for ~ 0.5 mm before exiting by penetrating the renal capsule again. The tip of the needle was inserted into one end of the dialysis probe and pulled together with the dialysis tube until the dialysis fiber was situated within the renal cortex. The flank wound was closed with clips and the inflow tube connected to a gas-tight syringe filled with lactated Ringer's infused at 3 μl/min (Harvard Apparatus, Inc., South Natick, MA), as done by others (14). To quantitate possible contamination of RIF with tubule contents caused by insertion of the microdialysis apparatus, we infused five animals having the microdialysis apparatus in place with a large amount (1 mCi) of ³H-inulin and compared late proximal tubule ³H-inulin concentration with that in RIF as determined by microdialysis. In vitro ³H-inulin recovery, evaluated by immersing dialysis membranes of five identically constructed probes into a beaker containing ³H-inulin, was 89%. RIF ³H-inulin concentration was 4.7% of that in the late proximal tubule, consistent with minimal leakage of tubule contents into RIF. Three consecutive 20-min collection periods were done in four each of control, (NH₄)₂SO₄, and Na₂SO₄ animals for RIF ET-1 measurements. In vitro ET-1 recoveries for four identically constructed probes in a beaker with ¹²⁵I-ET-1 (ICM Biomedicals, Irvine, CA) perfused as described was 59±2%. ET-1 in RIF dialysate was measured using a RIA kit (Peninsula Laboratories Inc., Belmont, CA) after disposable

column extraction (Sep-Pak C18; Millipore Corp., Milford, MA) pre-conditioned with methanol, H₂O, and acetic acid as described (15).

Microperfusion protocol. Animals were prepared for micropuncture of accessible distal tubules as described (16). This distal nephron segment is comprised of multiple epithelia (17) but we will hereafter refer to it as the "distal tubule" for simplicity. Distal tubules were perfused at the early distal flow rate measured in situ (6 nl/min) (18), calibrated in vitro, and verified in vivo (16). Transepithelial potential difference was measured after perfusate collection for each solution (16). An injected latex cast determined perfused tubule length after subsequent acid digestion of the kidney (16). Anaerobically obtained arterial (0.35 ml) and stellate vessel blood plasma (16) was analyzed for tCO₂ using flow-through fluorometry (see below) (19), and for pH, PCO₂, and electrolytes (16). Diet, but not drinking solution, was withheld the evening before studying micropunctured animals, yielding higher baseline HCO₃ reabsorption (20) and permitting differences in HCO₃ reabsorption to be more clearly seen.

Table I depicts perfusate composition. Standard perfusate HCO₃ and Cl⁻ were 5 and 40 mM, respectively, to approximate these anion concentrations in early distal tubule fluid of control animals (18). Solution 1 contained no HCO₃ to assess blood-to-lumen HCO₃ accumulation and to calculate an apparent transtubule HCO₃ permeability as done previously (16) and described below. Solution 2 was HCO₃⁻ and Cl⁻-free and contained 0.5 mM acetazolamide. Acetazolamide inhibits HCO₃ (16, 21) and H⁺ secretion (22) in the in vivo-perfused rat distal tubule. Thus, measuring luminal HCO₃ accumulation and voltage when perfusing with a zero HCO₃, zero Cl⁻, and acetazolamide-containing solution allows calculation of passive blood-to-lumen transepithelial HCO₃ permeability as done in our laboratory (16) and by others (23). Solution 2 was used in this way. Solution 3 contained 5 mM HCO₃ for measurement of net HCO₃ reabsorption and for calculation of luminal H⁺ secretion using the apparent transtubule HCO₃ permeability derived from perfusing with solution 1 as described (16). Solution 4 was identical to solution 3 except that it contained 10 rather than 5 mM NaHCO₃. This perfusing solution helped to determine if a minimum attainable HCO₃ in distal tubule fluid limited net HCO₃ reabsorption in the (NH₄)₂SO₄-ingesting animals as previously reported from this laboratory (3). All perfusing solutions contained raffinose to minimize fluid transport and permit more focused study of HCO₃ transport (16). Three selected distal tubules were perfused in each animal of each group. One distal tubule was perfused with solution 1, another with solution 2, and the third was perfused in paired fashion (16) with solutions 3 and 4 in random order. The order of perfusing solutions was random.

Analytical methods. Immediately after experiment termination, initial and collected perfusate, as well as stellate vessel plasma samples, were analyzed for inulin as done previously (16) and for tCO₂ using flow-through ultrafluorometry (19) as done previously (24). All tubule fluid and plasma tCO₂ were measured on the experimental

Table I. Perfusate Composition in Millimoles

	Solution			
	1	2	3	4
Na ⁺	61	61	61	61
K ⁺	4	4	4	4
Cl ⁻	40	0	40	40
HCO ₃	0	0	5	10
Gluconate	25	65	20	15
Acetazolamide	0	0.5	0	0
Raffinose	200	200	200	200

All solutions contained 0.5% FD&C green dye and were equilibrated with 6.7% CO₂.

day by comparing fluorescence of a 7–8-ml sample aliquot (corrected for a distilled H₂O blank run with each sample group) to a standard curve as previously described (24). This technique actually measures tCO₂, but we will refer to this measured value as HCO₃ for simplicity.

Calculations. Net HCO₃ transport was the difference between perfused and collected rates. Net HCO₃ reabsorption refers to net HCO₃ transport obtained when perfusing with initially HCO₃-containing solutions. Luminal HCO₃ accumulation describes net HCO₃ transport obtained when perfusing with initially HCO₃-free solutions. A positive value for HCO₃ transport indicates net HCO₃ movement out of the lumen (reabsorption) and a negative one indicates net HCO₃ movement into the lumen (secretion). A transtubule HCO₃ permeability (P_{HCO₃}) was calculated as done previously (16). The term “passive” HCO₃ permeability refers to that obtained when perfusing with zero HCO₃, zero Cl⁻, and acetazolamide-containing solution (solution 2). By contrast, the term “apparent” permeability refers to transtubule HCO₃ permeability obtained when perfusing with zero HCO₃, zero Cl⁻ solution that contained no acetazolamide (solution 1). Bicarbonate secretion was estimated during perfusion with HCO₃-containing solutions by calculating HCO₃ transport into the lumen using the apparent permeability derived from perfusing with the HCO₃-free solution (16). Thus, HCO₃ secretion = apparent permeability × transepithelial HCO₃ gradient corrected for transepithelial potential difference (16). The same apparent permeability (derived perfusing with solution 1 as discussed) was used to calculate HCO₃ secretion when perfusing with both the 5 mM (solution 3) and 10 mM (solution 4) HCO₃-containing solutions. By contrast, a unique transepithelial HCO₃ gradient was calculated for each HCO₃-containing solution using respective tubule HCO₃ concentrations measured when perfusing with that solution (16). The transepithelial potential difference used was the one measured when perfusing with the respective HCO₃-containing solution. H⁺ secretion was estimated during perfusion with the HCO₃-containing perfusates by subtracting calculated HCO₃ secretion from measured net HCO₃ reabsorption (16). This method for quantifying H⁺ secretion assumes that all HCO₃ transport from the lumen (absolute HCO₃ reabsorption) is mediated by luminal H⁺ secretion (22). Furthermore, this method underestimates H⁺ secretion to the extent that HCO₃ entering the perfusing solution during perfusion is subsequently reabsorbed. The perfusing solutions contained no NH₃/NH₄⁺ for reasons previously discussed (25). Fluid reabsorption (J_v) was the difference between perfused and collected flow rates. All transport values were corrected for perfused tubule length (in mm).

Statistical analysis. The number comprising the three experimental groups represents animals and not tubules. Each reported animal had at least one successful distal tubule perfusion with each perfusing solution. The Bonferroni method was used for *t* test comparison of means (*P* < 0.05) when multiple different comparisons of the same parameter were done in animals among the three animal groups.

Results

General response to dietary protocols. There were no body weight differences between H₂O (control), (NH₄)₂SO₄, and Na₂SO₄ animals at the start (255±8, 254±6, and 244±6 g, respectively) or end (268±9, 263±6, and 257±6 g, respectively) of the experimental period. Food intake was not different among groups. Ingested drinking solution volume was higher in (NH₄)₂SO₄ (37.1±2.9 ml/d) and Na₂SO₄ (33.2±2.0 ml/d) animals compared with control (21.3±1.8 ml/d, *P* < 0.02 vs. (NH₄)₂SO₄ and Na₂SO₄ groups). Similarly, urine flow was higher in (NH₄)₂SO₄ (21.1±1.9 ml/d) and Na₂SO₄ (18.2±1.5 ml/d) animals compared with control (11.4±0.9 ml/d, *P* < 0.03 vs. (NH₄)₂SO₄ and Na₂SO₄ groups).

Plasma and urine changes induced by drinking solutions. Plasma HCO₃ was not different from the respective baseline

value in (NH₄)₂SO₄ (25.6±1.4 vs. 25.9±1.4 mM, respectively, *P* = NS), control (25.5±1.6 vs. 25.7±1.5 mM, respectively, *P* = NS), or Na₂SO₄ (25.9±1.3 vs. 25.0±1.4 mM, respectively, *P* = NS) animals. Urine pH decreased compared with its baseline in (NH₄)₂SO₄ (5.32±0.06 vs. 6.17±0.05, respectively, *P* < 0.001) and Na₂SO₄ (5.45±0.06 vs. 6.10±0.05, respectively, *P* < 0.002), but not in control (6.15±0.05 vs. 6.16±0.05, respectively, *P* = NS) animals. Cumulative 7-d NAE was higher in (NH₄)₂SO₄ compared with control (42.2±3.1 vs. 19.0±1.8 meq/7 d, respectively, *P* < 0.002), but was not different from control in Na₂SO₄ (24.2±2.1 meq/7 d, *P* = 0.22) animals.

RIF ET-1 addition. Fig. 1 shows higher RIF ET-1 addition in (NH₄)₂SO₄ animals compared with control (480±51 vs. 293±32 fmol g kidney wt⁻¹ min⁻¹, respectively, *P* < 0.05), but that for Na₂SO₄ animals (275±23 fmol g kidney wt⁻¹ min⁻¹) was not different from control.

Effect of endothelin receptor inhibition on systemic blood pressure. Mean blood pressure of micropunctured animals was similar among bosentan-infused, BQ-123-infused, and baseline (NH₄)₂SO₄ (110±3, 108±3, and 115±4 mmHg, respectively), control (111±4, 112±3, and 116±3 mmHg, respectively), and Na₂SO₄ animals (107±3, 113±3, and 117±4 mmHg, respectively, *P* = NS).

Micropuncture data. Because the (NH₄)₂SO₄-induced increased urine NAE was accompanied by augmented RIF ET-1 addition, we investigated whether endothelin receptor antagonism in vivo influenced the augmented distal tubule acidification induced by dietary (NH₄)₂SO₄ (3). Plasma electrolyte and acid-base composition including arterial and stellate vessel plasma HCO₃ were not different among groups (data not shown). Table II depicts the effect of bosentan, a nonspecific endothelin receptor antagonist (10), and BQ-123, an ET_A selective antagonist (11), on distal tubule HCO₃ transport in situ. In previous studies, (NH₄)₂SO₄ animals had lower late distal fluid HCO₃ and lower HCO₃ delivery to this nephron segment than control (3). In the present studies, Table II shows that late distal tubule fluid HCO₃ was higher than baseline in (NH₄)₂SO₄ animals given bosentan but not BQ-123. Late distal tubule fluid HCO₃ was higher in bosentan-treated compared with baseline (NH₄)₂SO₄ animals despite similar fluid flows (2.0±0.2 vs. 1.8±0.2 nl/min, respectively, *P* = NS) and tubular fluid-to-plasma inulin ratios (14.1±1.8 vs. 14.8±1.9, respectively, *P* = NS). By contrast, early and late distal tubule HCO₃ delivery as well as net HCO₃ reabsorption were not different

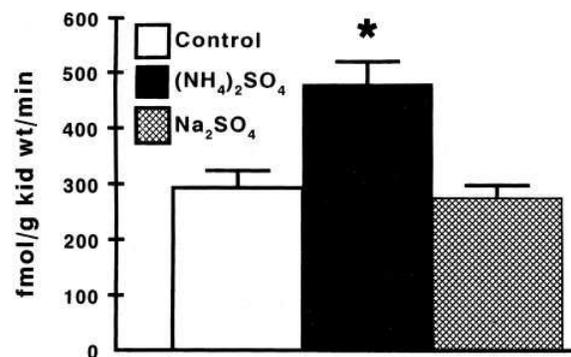


Figure 1. Renal interstitial fluid ET-1 addition in control (open bar), (NH₄)₂SO₄ (solid bar), and Na₂SO₄ (hatched bar) animals. **P* < 0.05 vs. control.

Table II. In Situ tCO₂ Transport in Distal Tubules

	ED HCO ₃	LD HCO ₃	ED HCO ₃ delivery	LD HCO ₃ delivery	Net HCO ₃ reabsorption
	mM		pmol/min		
(NH₄)₂SO₄ animals					
(NH ₄) ₂ SO ₄ (n = 4)	5.4±0.3	2.0±0.1	31.9±2.2	3.6±0.5	28.2±2.2
(NH ₄) ₂ SO ₄ + bosentan (n = 4)	5.3±0.3	3.1±0.2*	31.7±2.1	6.2±0.9	25.4±2.0
(NH ₄) ₂ SO ₄ + BQ-123 (n = 4)	5.1±0.3	1.8±0.1	31.0±2.1	3.4±0.4	27.6±2.2
Control animals					
H ₂ O (n = 5)	5.6±0.4	3.9±0.2	34.2±2.5	7.8±1.1	26.5±2.2
H ₂ O + bosentan (n = 4)	5.4±0.3	4.0±0.2	32.4±2.4	8.4±1.2	23.9±2.1
Na₂SO₄ animals					
Na ₂ SO ₄ (n = 4)	5.4±0.4	3.4±0.2	32.9±2.3	6.8±0.9	26.0±2.1
Na ₂ SO ₄ + bosentan (n = 4)	5.5±0.4	3.8±0.2	33.6±2.5	7.6±1.0	26.1±1.9

Values are means±SEM; ED, early distal tubule; LD, late distal tubule. Chemical designations represent drinking water content. *P < 0.05 vs. group without drug.

from baseline in bosentan- or BQ-123-infused (NH₄)₂SO₄ animals. Likewise, early distal tubule HCO₃ was similar among (NH₄)₂SO₄ animals. Table II shows that in situ distal tubule HCO₃ transport of bosentan-infused animals was not different from baseline in either control or Na₂SO₄ groups.

Decreased luminal HCO₃ induced by dietary acid might increase distal nephron NH₄⁺ secretion (28) and thereby increase nephron acid excretion as discussed (3). The bosentan-induced increase in late distal tubule HCO₃ in (NH₄)₂SO₄ animals is consistent with an endothelin role in the reduced late distal HCO₃ induced by dietary acid (3). The following studies examined if the bosentan-induced increase in late distal HCO₃ in situ was mediated by increased HCO₃ secretion, decreased H⁺ secretion, or both. Data from distal tubules perfused in paired fashion with HCO₃-free solution (solution 1) depicted in Table III were combined with those from paired perfusions with HCO₃-containing solutions 3 and 4 (5 and 10 mM HCO₃, respectively) in Tables V and VI to calculate components of net HCO₃ reabsorption (see Methods). In previous studies, (NH₄)₂SO₄ animals had lower luminal HCO₃ accumulation and lower apparent blood-to-lumen HCO₃ permeability than control when perfusing distal tubules with zero-HCO₃ solutions (3). Table III shows higher luminal HCO₃ accumulation and apparent blood-to-lumen HCO₃ permeability in distal tubules of bosentan-treated compared with baseline (NH₄)₂SO₄ animals, but these parameters were not different from baseline values in BQ-123-treated (NH₄)₂SO₄ animals.

To investigate if the increased luminal HCO₃ accumulation and apparent blood-to-lumen HCO₃ permeability in the bosentan-treated (NH₄)₂SO₄ animals was mediated by increased passive blood-to-lumen HCO₃ permeability (see Methods), distal tubules were perfused with an HCO₃⁻ and Cl⁻-free solution containing acetazolamide (solution 2). Table IV shows that when perfusing with this solution, luminal HCO₃ accumulation and apparent blood-to-lumen HCO₃ permeability were not different between bosentan-treated and baseline (NH₄)₂SO₄ animals. These data show that passive blood-to-lumen HCO₃ permeability is similar in bosentan-infused and baseline (NH₄)₂SO₄ animals.

The next microperfusion studies investigated the effect of endothelin receptor antagonism on net HCO₃ reabsorption as well as on calculated HCO₃ and H⁺ secretion in distal tubules of (NH₄)₂SO₄ animals. Table V shows that net HCO₃ reabsorption in bosentan- and BQ-123-treated (NH₄)₂SO₄ animals was not different from that of the baseline (NH₄)₂SO₄ group when their distal tubules were perfused with the 5-mM HCO₃ solution (solution 3). Fig. 2 shows that calculated HCO₃ secretion (see Methods) was higher in bosentan-infused compared with baseline (NH₄)₂SO₄ animals (-4.7±0.5 vs. -2.4±0.3 pmol mm⁻¹·min⁻¹, P < 0.03), but that for the BQ-123-infused (NH₄)₂SO₄ animals was not (-2.0±0.2 pmol mm⁻¹·min⁻¹, P = NS). By contrast, Fig. 3 shows that calculated H⁺ secretion was not different in the bosentan-infused compared with the baseline (NH₄)₂SO₄ animals (22.0±2.0 vs. 24.0±2.1 pmol mm⁻¹·s⁻¹).

Table III. Net Blood-to-Lumen HCO₃ Fluxes and Permeabilities in Distal Tubules of (NH₄)₂SO₄ Animals Perfused with Solution 1 (Zero HCO₃)

Tubule length mm	Flow rate nl/min		HCO ₃ mM			PD mV	J _{HCO3} pmol/mm·min ⁻¹	Apparent permeability × 10 ⁻⁷ cm ² /s
	Perfusion	J _v	Initial	Collected	log mean gradient			
(NH₄)₂SO₄ (n = 4)								
1.06±0.03	5.9±0.2	0.03±0.01	1.3±0.1	1.8±0.2	28.8±1.2	-13.1±1.2	-2.7±0.3	0.20±0.03
(NH₄)₂SO₄ + bosentan (n = 4)								
1.08±0.03	6.0±0.2	0.04±0.02	1.2±0.1	2.2±0.2	28.3±1.2	-10.2±1.2	-5.4±0.6*	0.39±0.04*
(NH₄)₂SO₄ + BQ-123 (n = 4)								
1.02±0.03	5.9±0.2	0.01±0.03	1.3±0.1	1.7±0.2	28.7±1.2	-12.8±1.2	-2.3±0.2	0.17±0.03

Values are means±SEM; chemical designations refer to content of drinking solution. *P < 0.05 vs. respective (NH₄)₂SO₄ group.

Table IV. Blood-to-Lumen HCO₃ Fluxes and Permeabilities in Distal Tubules of (NH₄)₂SO₄ Animals Perfused with Solution 2 (Zero HCO₃, Zero Cl⁻ with 0.5 mM Acetazolamide)

Tubule length mm	Flow rate nl/min		HCO ₃ mM			PD mV	J _{HCO₃} pmol/mm·min ⁻¹	Passive permeability × 10 ⁻⁷ cm ² /s	
	Perfusion	J _v	Initial	Collected	log mean gradient				
(NH ₄) ₂ SO ₄ (n = 4)	1.02±0.03	5.9±0.1	-0.01±0.04	1.3±0.1	2.0±0.2	28.7±0.8	-13.7±1.3	-4.1±0.5	0.31±0.04
(NH ₄) ₂ SO ₄ + bosentan (n = 4)	1.04±0.03	6.1±0.1	0.04±0.03	1.3±0.1	1.9±0.2	28.4±0.8	-12.5±1.2	-3.4±0.4	0.26±0.03

Values are means±SEM; chemical designations refer to content of drinking solution.

Table V. Bicarbonate Reabsorption by Distal Tubules of (NH₄)₂SO₄ Animals Perfused with Solution 3 (5 mM HCO₃)

Tubule length mm	Flow rate nl/min		HCO ₃ mM			PD mV	Net HCO ₃ reabsorption pmol/mm·min ⁻¹	
	Perfusion	J _v	Initial	Collected	log mean gradient			
(NH ₄) ₂ SO ₄ (n = 4)	1.02±0.03	5.9±0.1	0.03±0.02	5.6±0.3	1.9±0.2	26.6±0.9	-14.2±1.2	21.6±1.8
(NH ₄) ₂ SO ₄ + bosentan (n = 4)	1.01±0.03	6.0±0.1	0.01±0.03	5.8±0.3	3.1±0.3	25.5±0.8	-12.2±1.3	17.3±1.6
(NH ₄) ₂ SO ₄ + BQ-123 (n = 4)	1.07±0.03	6.1±0.2	0.02±0.01	5.6±0.3	1.7±0.2	26.5±1.0	-14.8±1.5	22.3±1.8

Values are means±SEM; chemical designations refer to content of drinking solution.

min⁻¹, respectively, *P* = NS). Calculated H⁺ secretion was also not different from baseline in BQ-123-infused (NH₄)₂SO₄ animals (24.3±2.2 pmol mm⁻¹·min⁻¹, *P* = NS vs. respective baseline value). Thus, increased HCO₃ secretion contributes to higher late distal tubule HCO₃ of bosentan-infused (NH₄)₂SO₄ animals.

The final perfusion studies in (NH₄)₂SO₄ animals examined if a minimal attainable HCO₃ might limit measured net HCO₃ reabsorption in distal tubules perfused with HCO₃-containing solutions, limiting calculated H⁺ secretion in these animals, and possibly concealing an effect of bosentan on H⁺ secretion. Distal tubules were perfused with solution 4, containing a higher initial HCO₃ (10 mM) than solution 3, as discussed earlier. The data are depicted in Table VI. In previous studies, net

HCO₃ reabsorption was higher in (NH₄)₂SO₄ compared with control animals when distal tubules of each were perfused with 10 mM HCO₃ solution (3). Table VI shows lower net HCO₃ reabsorption in distal tubules of bosentan-infused but not BQ-123-infused (NH₄)₂SO₄ animals. H⁺ and HCO₃ secretion for the 10-mM HCO₃ perfusions were calculated as for the 5-mM HCO₃ perfusions, using apparent permeability derived from the perfusion with solution 1 (see Methods). Fig. 4 shows that calculated HCO₃ secretion was higher in distal tubules of the bosentan-infused compared with the baseline (NH₄)₂SO₄ animals perfused with 10 mM HCO₃ (-3.9±0.4 vs. -2.0±0.2 pmol mm⁻¹·min⁻¹, *P* < 0.05), but that for the BQ-123-infused (NH₄)₂SO₄ animals was not (-1.7±0.2 pmol mm⁻¹·min⁻¹, *P* = NS). In contrast with the findings described when perfusing with the 5-mM HCO₃ solutions, Fig. 5 shows that bosentan-infused (NH₄)₂SO₄ animals had lower calculated H⁺ secretion compared with the baseline value (27.7±2.5 vs. 43.9±4.0 pmol mm⁻¹·min⁻¹, *P* < 0.05). H⁺ secretion in distal tubules of BQ-123-infused (NH₄)₂SO₄ animals was not different from the baseline value (42.9±4.2 pmol mm⁻¹·min⁻¹, *P* = NS). Thus,

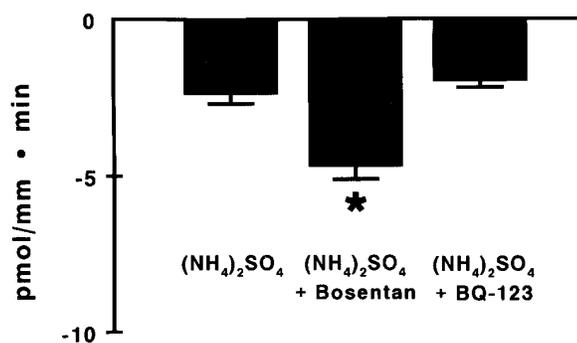


Figure 2. Bicarbonate secretion by distal tubules perfused with solution 3 (5 mM HCO₃) in (NH₄)₂SO₄-ingesting animals at baseline and after bosentan (10 mg/kg) or BQ-123 (1 mg/kg bolus followed by 0.1 mg kg⁻¹ min⁻¹) infusion. **P* < 0.05 vs. (NH₄)₂SO₄ (baseline) group.

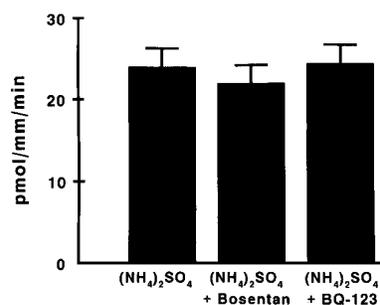


Figure 3. Proton secretion by distal tubules perfused with solution 3 (5 mM HCO₃) in (NH₄)₂SO₄-ingesting animals at baseline and after infusion of bosentan (10 mg/kg) or BQ-123 (1 mg/kg bolus followed by 0.1 mg kg⁻¹ min⁻¹).

Table VI. Bicarbonate Reabsorption by Distal Tubules ($(\text{NH}_4)_2\text{SO}_4$ Animals Perfused with Solution 4 (10 mM HCO_3^-)

Tubule length mm	Flow rate nl/min		HCO_3^- mM			PD mV	Net HCO_3^- reabsorption pmol/mm·min ⁻¹
	Perfusion	J_v	Initial	Collected	log mean gradient		
$(\text{NH}_4)_2\text{SO}_4$ ($n = 3$)							
0.96±0.03	5.9±0.2	0.01±0.03	11.3±0.2	4.5±0.2*	22.3±1.0	-15.0±1.4	41.9±3.8
$(\text{NH}_4)_2\text{SO}_4$ + bosentan ($n = 4$)							
1.03±0.04	6.2±0.3	0.05±0.04	11.2±0.3	7.3±0.3*	21.7±1.1	-12.9±1.3	23.8±2.5*
$(\text{NH}_4)_2\text{SO}_4$ + BQ-123 ($n = 3$)							
0.98±0.03	6.0±0.2	0.04±0.02	11.1±0.2	4.4±0.2	22.3±1.1	-15.1±1.5	41.2±4.2

Values are means±SEM; chemical designations refer to content of drinking solution. * $P < 0.05$ vs. respective $(\text{NH}_4)_2\text{SO}_4$ group.

Table VII. Net Blood-to-Lumen HCO_3^- Fluxes and Permeabilities in Distal Tubules of Control Animals Perfused with Solution 1 (Zero HCO_3^-)

Tubule length mm	Flow rate nl/min		HCO_3^- mM			PD mV	$J_{\text{HCO}_3^-}$ pmol/mm·min ⁻¹	Apparent permeability $\times 10^{-7}$ cm ² /s
	Perfusion	J_v	Initial	Collected	log mean gradient			
H_2O ($n = 5$)								
1.03±0.03	5.9±0.2	0.01±0.03	1.3±0.1	2.3±0.2	31.7±1.2	-12.9±1.3	-5.7±0.8	0.39±0.05
H_2O + bosentan ($n = 4$)								
1.05±0.04	5.9±0.1	0.02±0.02	1.2±0.1	2.4±0.2	31.7±1.3	-12.4±1.2	-6.6±0.7	0.44±0.05

Values are means±SEM; chemical designations refer to content of drinking solution.

decreased H^+ secretory capacity contributes to higher HCO_3^- at the late distal tubule in situ of bosentan-infused $(\text{NH}_4)_2\text{SO}_4$ animals.

We next examined the effect of bosentan on HCO_3^- transport in control animals. Table VII shows no difference in luminal HCO_3^- accumulation or apparent permeability between bosentan-infused and baseline controls perfused with solution 1 (zero HCO_3^-). Because differences in net HCO_3^- reabsorption between $(\text{NH}_4)_2\text{SO}_4$ animals and control were evident perfusing with the 10 but not 5 mM HCO_3^- solution, the 10-mM solution (solution 4) was perfused in distal tubules of control animals to determine if bosentan influenced net HCO_3^- reabsorption and/or its components ($\text{HCO}_3^-/\text{H}^+$ secretion) in con-

rol animals. Table VIII shows that net HCO_3^- reabsorption was not different between bosentan-infused and baseline control animals perfused with 10 mM HCO_3^- . Calculated secretion of HCO_3^- (-5.0 ± 0.6 vs. -4.4 ± 0.5 pmol mm⁻¹·min⁻¹, $P = \text{NS}$) and H^+ (20.2 ± 2.0 vs. 23.2 ± 2.2 pmol mm⁻¹·min⁻¹, $P = \text{NS}$) were also not different in bosentan-infused and baseline controls, respectively. Thus, endothelin receptor antagonism did not affect distal tubule acidification in control animals.

The final series of microperfusion studies determined if bosentan influenced distal tubule HCO_3^- transport in Na_2SO_4 animals, a group whose urine flow was higher than control but comparable to that for $(\text{NH}_4)_2\text{SO}_4$ animals. This higher urine flow might itself increase urine ET-1 excretion (9) and thereby influence distal tubule acidification independent of dietary acid intake. Table IX shows no difference in luminal HCO_3^- accumulation or apparent permeability between bosentan-infused and baseline Na_2SO_4 animals perfused with solution 1 (zero HCO_3^-). As with the control animals, the 10-mM HCO_3^- solution (solution 4) was used in distal tubules of Na_2SO_4 animals

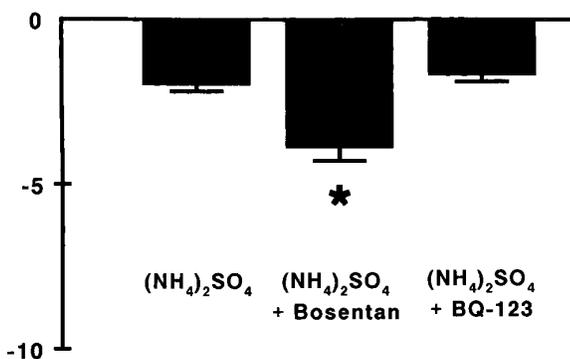


Figure 4. Bicarbonate secretion by distal tubules perfused with solution 4 (10 mM HCO_3^-) in $(\text{NH}_4)_2\text{SO}_4$ -ingesting animals at baseline and after infusion of bosentan (10 mg/kg) or BQ-123 (1 mg/kg bolus followed by 0.1 mg kg⁻¹ min⁻¹). * $P < 0.05$ vs. $(\text{NH}_4)_2\text{SO}_4$ (baseline) group.

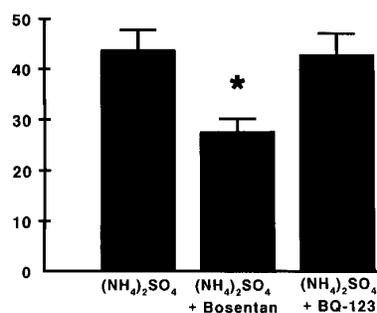


Figure 5. Proton secretion by distal tubules perfused with solution 4 (10 mM HCO_3^-) in $(\text{NH}_4)_2\text{SO}_4$ -ingesting animals at baseline and after infusion of bosentan (10 mg/kg) or BQ-123 (1 mg/kg bolus followed by 0.1 mg kg⁻¹ min⁻¹). * $P < 0.05$ vs. $(\text{NH}_4)_2\text{SO}_4$ (baseline) group.

Table VIII. Bicarbonate Reabsorption by Distal Tubules of Control Animals Perfused with Solution 4 (10 mM HCO₃)

Tubule length mm	Flow rate nl/min		HCO ₃ mM			PD mV	Net HCO ₃ reabsorption pmol/mm·min ⁻¹
	Perfusion	J _v	Initial	Collected	log mean gradient		
H ₂ O (n = 4)							
1.04±0.03	5.9±0.1	0.01±0.03	10.6±0.5	7.3±0.3	24.5±1.3	-13.2±1.5	18.8±1.9
H ₂ O + bosentan (n = 4)							
1.02±0.03	5.9±0.1	0.01±0.03	10.3±0.4	7.7±0.3	24.5±1.3	-12.8±1.2	15.1±1.5

Values are means±SEM; chemical designations refer to content of drinking solution.

Table IX. Net Blood-to-Lumen HCO₃ Fluxes and Permeabilities in Distal Tubules of Na₂SO₄ Animals Perfused with Solution 1 (Zero HCO₃)

Tubule length mm	Flow rate nl/min		HCO ₃ mM			PD mV	J _{HCO₃} pmol/mm·min ⁻¹	Apparent permeability × 10 ⁻⁷ cm ² /s
	Perfusion	J _v	Initial	Collected	log mean gradient			
Na ₂ SO ₄ (n = 4)								
0.93±0.04	5.9±0.2	-0.03±0.01	1.3±0.1	2.0±0.2	30.3±1.1	-16.2±1.5	-4.5±0.6	0.34±0.05
Na ₂ SO ₄ + bosentan (n = 4)								
0.97±0.04	6.1±0.2	0.04±0.02	1.4±0.1	2.2±0.2	30.0±1.1	-15.1±1.3	-5.2±0.7	0.38±0.05

Values are means±SEM; chemical designations refer to content of drinking solution.

to determine if bosentan influenced net HCO₃ reabsorption and/or its components in this group. Table X shows that net HCO₃ reabsorption was not different between bosentan-infused and baseline Na₂SO₄ animals. Calculated secretion of HCO₃ (-3.8±0.5 vs. -3.4±0.4 pmol mm⁻¹·min⁻¹, P = NS) and H⁺ (23.8±2.2 vs. 29.4±2.6 pmol mm⁻¹·min⁻¹, P = NS) were also not different in bosentan-infused and baseline Na₂SO₄ animals, respectively. Thus, endothelin receptor antagonism did not affect distal tubule acidification in Na₂SO₄ animals.

Effect of endothelin receptor inhibition on NAE. Because bosentan decreased acidification in the distal tubule of (NH₄)₂SO₄ animals, we investigated if this receptor antagonist reduced NAE. 12-h NAE after administration of the same bosentan dose (10 mg/kg i.v.) that had been given to micro-punctured animals was not different in bosentan-infused compared with vehicle-infused (NH₄)₂SO₄ (3.2±0.5 vs. 4.1±0.6 meq/12 h, respectively, P = NS), control (1.7±0.3 vs. 1.7±0.2 meq/12 h, respectively, P = NS), or Na₂SO₄ (1.7±0.2 vs. 1.8±0.2 meq/12 h, respectively, P = NS) animals.

Discussion

Many investigations employing a variety of techniques have defined effector mechanisms by which the kidney increases acidification in response to dietary acid (26). Such investigations show an important role of increased distal nephron acidification in this response (1–3, 27–29). The distal tubule of animals ingesting dietary acid have augmented H⁺ secreting capacity (1–3) and reduced HCO₃ delivery to the terminal distal nephron (3, 30), the latter facilitating increased titration of non-HCO₃ buffers (28, 31). Recent studies from our laboratory show that reduced distal tubule HCO₃ secretion contributes to the decreased terminal distal nephron HCO₃ delivery induced by dietary acid (3). Thus, dietary acid alters both components of distal tubule net HCO₃ reabsorption (H⁺/HCO₃ secretion) in a direction that increases acidification, but the immediate stimulus that induces this response is not known. Much less is understood about the mechanisms that induce distal nephron epithelia to respond in this way to a dietary acid challenge. Dietary acid-induced alterations in body fluid acid-

Table X. Bicarbonate Reabsorption by Distal Tubules of Na₂SO₄ Animals Perfused with Solution 4 (10 mM HCO₃)

Tubule length mm	Flow rate nl/min		HCO ₃ mM			PD mV	Net HCO ₃ reabsorption pmol/mm·min ⁻¹
	Perfusion	J _v	Initial	Collected	log mean gradient		
Na ₂ SO ₄ (n = 4)							
1.01±0.05	6.1±0.1	0.05±0.03	10.9±0.5	6.7±0.4	23.1±1.3	-15.9±1.5	25.6±2.3
Na ₂ SO ₄ + bosentan (n = 4)							
0.98±0.03	5.8±0.2	-0.04±0.02	10.7±0.6	7.2±0.4	22.8±1.3	-15.8±1.6	20.4±1.8

Values are means±SEM; chemical designations refer to content of drinking solution.

base parameters might increase distal nephron acidification directly. Although changes in environmental acid–base parameters can induce predictable alterations in acidification of renal epithelia studied *in vitro* (32, 33), it is clear that sustained changes in plasma (3, 24) or intracellular (4) acid–base parameters are not necessary to maintain altered renal epithelial acidification (3, 4, 24). Alternative or additional mechanisms include diet-induced modification of the level and/or activity of secretory substances that modify renal epithelial acidification. The present studies tested the hypothesis that endothelin mediates increased distal tubule acidification induced by dietary acid. This hypothesis derives from studies showing that ET-1 increases NHE-3 activity in cultured renal epithelial cells (8) and decreases distal tubule HCO_3^- secretion induced by dietary HCO_3^- (7). The data show that animals given dietary acid have greater ET-1 addition to RIF. Furthermore, B- but not A-type endothelin receptor inhibition blunts decreased HCO_3^- secretion and increased H^+ -secreting capacity induced by dietary acid. Endothelin receptor inhibition had no measurable effect on distal tubule acidification in control animals, suggesting that endothelin contributes less to the “tonic” level of distal tubule acidification under control conditions. The data support the hypothesis that endothelin mediates increased distal tubule acidification induced by dietary acid.

Although the endothelins were initially noted for their vasoactive effects (34), these agents also modulate epithelial transport by inhibiting the amiloride-sensitive Na^+ channel (35) and ADH-mediated H_2O reabsorption (36) in collecting tubules. ET-1 also stimulates the Na^+/H^+ exchanger in renal cortical membrane vesicles (37) and the NHE-3 isoform in renal epithelial cells (8), supporting a possible endothelin role in modulating renal acidification *in vivo*. The NHE performs much of the H^+ secretion in the rat distal tubule accessible to micropuncture (38). ET-1 also inhibits agonist-stimulated increases in cellular cyclic AMP levels in renal epithelium (36), a cellular second messenger whose increase is associated with augmented HCO_3^- secretion in cortical collecting tubules (39) and with inhibited NHE activity in renal brush border membranes (40). Preliminary studies show that ET-1 decreases distal tubule HCO_3^- secretion induced by dietary HCO_3^- (7), an important response to this dietary maneuver (24). By contrast, ET-1 inhibited net HCO_3^- reabsorption in proximal straight tubules (41), suggesting distinct ET-1 effects on acidification in this nephron segment. The present studies show that endothelin receptor inhibition blunts decreased HCO_3^- secretion and increased H^+ secretion in the distal tubule induced by dietary acid (3). The data suggest that endogenous endothelins mediate the decreased HCO_3^- secretion and increased H^+ secretion induced by dietary acid.

The present studies show that dietary acid increases ET-1 addition to RIF, a fluid compartment in direct communication with basolateral surfaces of cortical renal epithelium. This anatomical arrangement would permit endothelins secreted into RIF to modulate cortical epithelial transport through endothelin receptors on the surface of cultured canine cells of distal nephron origin (42). Furthermore, rat cortical collecting tubules contain mRNA for the B-type endothelin receptor (43), which the present studies suggest mediates the described actions of endogenous endothelins. Endothelins in the RIF might derive from collecting tubule epithelium (5) or endothelium of the renal microvasculature (6, 44). The latter is separated from distal tubules *in vivo* by only a very narrow interstitial space

(45), permitting paracrine or autocrine communication among cell types. In addition, microdissected cortical collecting tubules contain ET-1 mRNA (43, 46), and ET-1 is on the endothelial surfaces of peritubular capillaries (44). The present studies show that dietary acid increases RIF ET-1 addition and suggest that the added ET-1 subsequently increases distal tubule acidification.

Calculated HCO_3^- and H^+ secretion were not different in bosentan-infused control animals, in contrast with the $(\text{NH}_4)_2\text{SO}_4$ animals in which bosentan increased HCO_3^- secretion and decreased H^+ secretory capacity. Although a higher bosentan dose might influence control $\text{HCO}_3^-/\text{H}^+$ secretion, the data suggest that endogenous endothelins have a greater influence on basal distal tubule acidification in acid-ingesting compared with control animals. By contrast, endogenous endothelins apparently contribute less than intrinsic, neural, or other autocrine/paracrine mechanisms to basal distal tubule acidification. Nevertheless, the same bosentan dose that reduced distal nephron acidification in $(\text{NH}_4)_2\text{SO}_4$ animals did not reduce augmented NAE in these animals. This suggests less endothelin effect on acidification and/or less sensitivity to the administered bosentan dose in more terminal nephron segments.

The present studies support that endogenous endothelins inhibit distal tubule HCO_3^- secretion and stimulate H^+ secretion in this nephron segment, but do not indicate the cellular signaling mechanisms that mediate these effects. Increased cellular cAMP levels are associated with stimulated HCO_3^- secretion by the collecting tubule (39) and with inhibited Na^+/H^+ exchange in brush border membranes (40). In addition, ET-1 inhibits agonist-induced increases in cortical collecting tubule cAMP (36), complementing the ET-1 effects on distal tubule HCO_3^- secretion. Furthermore, prostacyclin (PGI_2), an agent that increases cellular cAMP levels in distal nephron epithelia (47), increases distal tubule HCO_3^- secretion (48). Yet, ET-1 also stimulates Ca^{2+} release from internal stores and its entry into cortical collecting duct cells (49), consistent with activation of phospholipase C (50). In addition, activation of the B-type endothelin receptor leads to generation of nitric oxide through a tyrosine kinase–dependent and Ca^{2+} /calmodulin-dependent pathway (51). Thus, ET-1 might alter $\text{HCO}_3^-/\text{H}^+$ secretion by these and possibly other mechanisms.

In summary, the present studies show that reduced HCO_3^- secretion and increased H^+ secretory capacity in the distal tubule induced by dietary acid is associated with increased ET-1 addition to RIF. Furthermore, inhibition of B- but not A-type endothelin receptors blunts the reduced HCO_3^- secretion and increased H^+ secretory capacity induced by dietary acid, but does not affect distal tubule acidification in control animals. The data suggest that endogenous endothelins mediate increased distal tubule acidification induced by dietary acid but contribute less to basal acidification in this segment.

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