

# Increased Endothelin Activity Mediates Augmented Distal Nephron Acidification Induced by Dietary Protein

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**Abstract.** The hypothesis that increased dietary protein augments distal nephron acidification and does so through an endothelin (ET-1)-dependent mechanism was tested. Munich-Wistar rats that ate minimum electrolyte diets of 50% (HiPro) and 20% (CON) casein-provided protein, the latter comparable to standard diet, were compared. HiPro versus CON had higher distal nephron net  $\text{HCO}_3^-$  reabsorption by *in vivo* microperfusion ( $37.8 \pm 3.2$  versus  $16.6 \pm 1.5$  pmol/mm per min;  $P < 0.001$ ) as a result of higher  $\text{H}^+$  secretion ( $41.3 \pm 4.0$  versus  $23.0 \pm 2.1$  pmol/mm per min;  $P < 0.002$ ) and lower  $\text{HCO}_3^-$  secretion ( $-3.5 \pm 0.4$  versus  $-6.4 \pm 0.8$  pmol/mm per min;  $P < 0.001$ ). Perfusion with  $\text{H}^+$  inhibitors support that increased  $\text{H}^+$  secretion was mediated by augmented  $\text{Na}^+/\text{H}^+$  exchange and  $\text{H}^+$ -ATPase activity without augmented  $\text{H}^+/\text{K}^+$ -ATPase activity. HiPro versus CON had higher levels of

urine ET-1 excretion, renal cortical ET-1 addition to microdialysate *in vivo*, and renal cortical ET-1 mRNA, consistent with increased renal ET-1 production. Oral bosentan, an ET A/B receptor antagonist, decreased distal nephron net  $\text{HCO}_3^-$  reabsorption ( $22.4 \pm 1.9$  versus  $37.8 \pm 3.2$  pmol/mm per min;  $P < 0.001$ ) as a result of lower  $\text{H}^+$  secretion ( $28.4 \pm 2.4$  versus  $41.3 \pm 4.0$  pmol/mm per min;  $P < 0.016$ ) and higher  $\text{HCO}_3^-$  secretion ( $-6.0 \pm 0.7$  versus  $-3.5 \pm 0.4$  pmol/mm per min;  $P < 0.006$ ). The  $\text{H}^+$  inhibitors had no additional effect in HiPro ingesting bosentan, supporting that ET mediated the increased distal nephron  $\text{Na}^+/\text{H}^+$  exchange and  $\text{H}^+$ -ATPase activity in HiPro. Increased dietary protein augments distal nephron acidification that is mediated through an ET-sensitive increase in  $\text{Na}^+/\text{H}^+$  exchange and  $\text{H}^+$ -ATPase activity.

The routine acid challenges to systemic acid-base status faced by humans are modest compared with the large acid loads administered to animals in most experimental protocols. Augmented distal rather than proximal nephron acidification is the predominant renal regulatory response in experimental animals to modest dietary acid loads induced by acid-producing mineral salts (1,2). Augmented distal nephron acidification induced by dietary acid is mediated by multiple mechanisms, including (1) increased net  $\text{HCO}_3^-$  reabsorption (3), consistent with increased  $\text{H}^+$  secretion; (2) reduced  $\text{HCO}_3^-$  delivery to the terminal distal nephron (4) that facilitates  $\text{NH}_4^+$  secretion (5) and permits secreted  $\text{H}^+$  to effect acid excretion rather than  $\text{HCO}_3^-$  reclamation; and (3) decreased distal nephron  $\text{HCO}_3^-$  secretion (1) mediated by endogenous endothelins (ET) (2).

In contrast with the acid-producing mineral salts that are most commonly used to induce an acid challenge in experimental protocols, increased intake of dietary protein that contains acid-producing amino acids constitutes the acid challenge that humans more routinely face. Intake of acid-producing amino acids increases systemic acid production and urine net

acid excretion (6), but its effect on distal nephron acidification or its hormonal and/or transport mediators are not known. Recognizing that ET mediate increased distal nephron acidification induced by modest dietary acid loads as a result of intake of acid-producing mineral salts (1,2), the present studies tested the hypothesis that increased intake of acid-producing amino acids as dietary protein increases distal nephron acidification and that this increased acidification is mediated by enhanced ET activity.

## Materials and Methods

### Animals and Diet Protocol

Male and female Munich-Wistar rats (Harlan Sprague-Dawley, Houston, TX) that weighed 200 to 220 g ate standard rat chow (Prolab RMH 2500 with 23% protein) for 1 wk (week 0), then ate a custom minimum electrolyte diet with protein as purified high nitrogen casein (ICN Nutritional Biochemicals, Cleveland, OH) for 3 wk (weeks 1, 2, and 3). High-protein rats (HiPro) ate custom diet with 50% protein, and controls (CON) ate 20%. In preliminary studies, similar-weight rats ate  $24.6 \pm 0.9$  and  $27.1 \pm 1.2$  g/d, respectively ( $n = 4$ ,  $P = 0.15$ ), and so all rats received 24 g/d diet to ensure similar diet intake. Some animals received bosentan (Hoffman-LaRoche, Basel, Switzerland), a nonpeptide ET A/B receptor antagonist (7), mixed with study diet at 100 mg/kg body wt per d and so was completely ingested. This oral dose blocks action of pressor doses of intravenous big ET-1 for >24 h (7). All drank distilled  $\text{H}_2\text{O}$  except for a separate CON group that was given 4.0% dextrose drinking solution to approximate the increased urine output associated with the HiPro diet. Animals drank *ad libitum*.

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### Urine Net Acid and ET-1 Excretion

We measured daily excretion of urine net acid (NAE) (8) and ET-1 (9) in a 24-h sample collected on days 7 (week 0), 14 (week 1), 21 (week 2), and 28 (week 3) of the protocol from eight each of HiPro and CON in metabolic cages. We examined the effect of ET receptor blockade with bosentan on urine NAE in paired and separate groups of eight each (four with and four without drug) of HiPro and CON. NAE was the mean for each animal group.

### Arterial Blood Parameters

We measured pH, PCO<sub>2</sub>, calculated [HCO<sub>3</sub>]<sup>-</sup> (IRMA Blood Analysis System, Diametrics Medical, St. Paul, MN), and total CO<sub>2</sub> (TCO<sub>2</sub>) by ultrafluorometry (see below) in 1.0 ml of blood from a chronic carotid arterial catheter in eight each of awake, gently restrained, and calm HiPro and CON at weeks 1 and 3 to assess the effects of HiPro on plasma acid-base parameters. We also measured arterial BP through this chronic arterial catheter as done previously (9).

### Microdialysis Technique for Measurement of Renal Cortical Fluid ET-1

Renal cortical fluid ET-1 addition was measured using microdialysis of the renal cortex as done in our laboratory (10) at weeks 0, 1, 2, and 3. Three consecutive 20-min collection periods were done in four each of HiPro, CON, and CON + 4.0% dextrose animals for microdialysate ET-1 measurements.

### Micropuncture Protocol

Animals were prepared for micropuncture of accessible distal tubules (9) at weeks 1 and 3. *In situ* early distal flow rate for HiPro and CON was 9.4 ± 0.7 (*n* = 6) and 6.4 ± 0.4 nl/min (*n* = 8), respectively. Separate superficial distal nephrons of HiPro and CON were each perfused 9 and 6 nl/min with a Hampel pump to approximate respective *in situ* flow rates. We measured distal tubule transepithelial potential difference to calculate blood-to-lumen HCO<sub>3</sub> permeability (9). After weighing the micropunctured (left) kidney, perfused nephron length was determined by measuring the length of a latex cast injected after micropuncture, recovered after acid digestion of the kidney (9). We measured [HCO<sub>3</sub>]<sup>-</sup> in stellate vessel plasma to determine peritubular blood-to-lumen HCO<sub>3</sub> gradient for calculating transepithelial H<sup>+</sup>/HCO<sub>3</sub> passive permeability (9). Diet but not H<sub>2</sub>O was withheld the evening before micropuncture to yield higher baseline HCO<sub>3</sub> reabsorption (11), as done previously (9).

The perfusion solutions used are in Table 1. Solution 1 contained 5 mM HCO<sub>3</sub><sup>-</sup> and 40 mM Cl<sup>-</sup> to approximate their concentrations at the early distal nephron *in situ* (12). Solution 2 contained Cl<sup>-</sup> but no HCO<sub>3</sub><sup>-</sup> to measure Cl<sup>-</sup>-dependent luminal HCO<sub>3</sub><sup>-</sup> accumulation and to

calculate an “apparent” blood-to-lumen H<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> permeability (9). Solution 3 was HCO<sub>3</sub><sup>-</sup>- and Cl<sup>-</sup>-free and contained 0.5 mM acetazolamide to inhibit transtubule H<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> transport and was used to determine “passive” blood-to-lumen H<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> permeability (9,13). We used this “passive” permeability determined using solution 3 to calculate passive blood-to-lumen HCO<sub>3</sub><sup>-</sup> secretion when perfusing with the HCO<sub>3</sub><sup>-</sup>-containing solution 1 (9). We used the “apparent” blood-to-lumen H<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> permeability determined from perfusing with solution 2 to calculate “total” HCO<sub>3</sub><sup>-</sup> secretion when perfusing with HCO<sub>3</sub><sup>-</sup>-containing solution 1 (9,13). We subtracted calculated “passive” HCO<sub>3</sub><sup>-</sup> secretion from calculated “total” HCO<sub>3</sub><sup>-</sup> secretion to obtain “net” HCO<sub>3</sub><sup>-</sup> secretion when perfusing with solution 1 (9,13). The HCO<sub>3</sub><sup>-</sup> secretion reported herein is the “net” HCO<sub>3</sub><sup>-</sup> secretion that excludes the passive HCO<sub>3</sub><sup>-</sup> secretion calculated as described above. Distal nephron H<sup>+</sup> secretion was calculated by subtracting the calculated “net” HCO<sub>3</sub><sup>-</sup> secretion (a negative value) from the measured net HCO<sub>3</sub><sup>-</sup> reabsorption (HCO<sub>3</sub><sup>-</sup> perfused into the distal nephron minus HCO<sub>3</sub><sup>-</sup> collected) (13). All perfusing solutions contained raffinose to minimize fluid transport and gluconate substituted for Cl<sup>-</sup> when necessary (9). Each surface distal nephron was perfused with each perfusing solution in the following order: 1, 2, 3. Previous studies that conducted random perfusions of these solutions showed that the order of perfusing solutions did not affect calculations of the components of distal nephron HCO<sub>3</sub><sup>-</sup> reabsorption (9).

### Identification of the H<sup>+</sup> Transport Mediators of HiPro-Induced Changes in Distal Nephron Acidification

We compared the net decrease in distal nephron H<sup>+</sup> secretion in response to specific H<sup>+</sup> transport inhibitors in HiPro *versus* CON to determine the contribution of Na<sup>+</sup>/H<sup>+</sup> exchange (EIPA, 10<sup>-5</sup> M), H<sup>+</sup>-ATPase (bafilomycin, 10<sup>-7</sup> M), and H<sup>+</sup>,K<sup>+</sup>-ATPase (Sch 28080, 10<sup>-5</sup> M) as done previously in our laboratory (14). Greater inhibitor-induced decrease in H<sup>+</sup> secretion in HiPro *versus* CON determined increased activity of the H<sup>+</sup>-transport inhibited by that compound (14).

### Qualitative Comparison of ET-1 mRNA Expression

**Total RNA Extraction and Reverse Transcription-PCR.** After treatment, kidneys were removed from anesthetized rats. Pieces of tissues were immediately frozen in liquid nitrogen and stored at -80°C until use.

**Total RNA Isolation.** Total RNA was isolated using 1 ml of TRI-Reagent (Molecular Research Center, Cincinnati, OH) for 50 mg of tissue, a commercial variant of the guanidium thiocyanate-phenol-chloroform reagent, using the manufacturer's suggested protocol (15). The resulting RNA was dissolved in DNase/Rnase-free water and stored at -20°C until use. Only RNA preparations whose A<sub>260</sub>/A<sub>280</sub> ratio exceeded 1.6 were analyzed further. The RNA quality was assessed by running the samples in a 1% formaldehyde agarose gel following standard protocol.

**Reverse Transcription.** We then performed reverse transcription with 2 μg of RNA, preheated 5 min at 65°C, in a final volume of 20 μl that contained 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 0.2 mmol of dNTP (Roche, Indianapolis, IN), 10 mM dithiothreitol, 1 mg of oligo(dT)<sub>12-18</sub> primers (Roche), 40 units of RNasin (Promega, Madison, WI), and 200 units of Moloney murine leukemia virus reverse transcriptase (Promega). After 1 h at 37°C, the enzyme was inactivated by boiling (10 min at 95°C).

**PCR.** Specific oligonucleotide primers (5'-CTCTGCTGTTTGTGGCTTTC-3' and 5'-GTCTGTGGTCTTTGTGGGA-3' for sense and antisense primers, respectively) were designed to hybridize the rat

Table 1. Perfusate composition (mM)

	Solution 1	Solution 2	Solution 3
Na <sup>+</sup>	61	61	61
K <sup>+</sup>	4	4	4
Cl <sup>-</sup>	40	40	0
HCO <sub>3</sub> <sup>-</sup>	5	0	0
Gluconate	20	25	65
Acetazolamide	0	0	0.5
Raffinose	200	200	200

ET-1 (rET-1) mRNA using Vector NTI 7 (InforMax, Frederick, MD). rET-1 cDNA amplification was carried out as follows: 1  $\mu$ l of the reverse-transcribed mixture was added to the PCR mixture that contained 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM MgCl<sub>2</sub>, 200  $\mu$ M of dNTP, 400 nmol of each primer, and 2.5 units of TaqDNA polymerase (Roche) up to a final volume of 50  $\mu$ l. After 2 min at 94°C, samples were submitted to 30 cycles under the following conditions: 45 s at 94°C, 45 s at 48°C (specific annealing temperature for the rET-1 primers), and 45 s at 72°C. After the final cycle, an additional elongation period of 7 min was performed at 72°C.

**Real-Time PCR.** Because rat kidneys express very little ET-1 mRNA, we performed a real-time PCR using the LightCycler apparatus (Roche) after the conventional PCR. The SYBR Green, which has a high affinity for double-stranded DNA (dsDNA) and exhibits enhancement of fluorescence upon binding to the dsDNA, was chosen as the fluorescent dye. Each reaction contained 2  $\mu$ l of cDNA from the conventional PCR, 3 mM MgCl<sub>2</sub>, 0.5 mM of each primer, and 1 $\times$  of FastStart DNA Master SYBR Green I mix (Roche) in a 20- $\mu$ l final volume. Samples were then placed in the LightCycler instrument in duplicate and underwent the following thermal cycling profile: cDNA was denatured by a preincubation of 30 s at 95°C, and the template was amplified for 35 cycles of (1) denaturation for 0 s at 95°C, (2) annealing at 48°C for 10 s, and (3) extension at 72°C for 25 s. The increase in fluorescence, dependent on the initial template concentration, was acquired after each extension phase at 83°C, a temperature above the T<sub>m</sub> of the primer dimers and below the T<sub>m</sub> of the specific PCR product, thus minimizing acquisition of nonspecific fluorescence intensities. After amplification, a melting curve was generated by cooling the samples to 55°C for 30 s and slowly heating the samples at 0.1°C/s to 95°C while the fluorescence was measured continuously. The LightCycler run was concluded with a 40°C incubation for 30 s. Groups were qualitatively compared, with earlier amplification indicating greater number of ET-1 mRNA copies. Product identity was confirmed by sequence analysis and electrophoresis on a 1% agarose gel stained with ethidium bromide (Expected PCR product size, 290 bp).

### Analytical Methods

Immediately after experiment termination, initial and collected perfusate, as well as stellate vessel plasma samples, were analyzed for inulin (9) and for TCO<sub>2</sub> using flow-through ultrafluorometry (16) as described previously (17). All tubule fluid and plasma TCO<sub>2</sub> were measured on the experimental day by comparing fluorescence of a 7- to 8-nl sample aliquot (corrected for a distilled H<sub>2</sub>O blank run with each sample group) to a standard curve as described previously (17). This technique actually measures TCO<sub>2</sub>, but we refer to this measured value as HCO<sub>3</sub> for simplicity.

Microdialysate and urine [ET-1] was measured using a RIA kit (Peninsula Laboratories, Belmont, CA) after disposable column extraction (Sep-Pak C18, Milford, MA) preconditioned with methanol, H<sub>2</sub>O, and acetic acid as done previously (10) at weeks 0, 1, 2, and 3.

### Statistical Analyses

Data were expressed as means  $\pm$  SEM. Paired perfusions of the same tubule were compared using paired *t* test; otherwise, ANOVA was used for multiple group comparisons. We used the Bonferroni method for multiple comparisons (*P* < 0.05) of the same parameter among groups.

## Results

### Effect of HiPro on Animal/Kidney/Tubule Growth and Urine Volume

HiPro and CON had similar body weight at week 0 (214  $\pm$  4 versus 216  $\pm$  5 g, respectively), but HiPro gained more weight (159  $\pm$  4 versus 114  $\pm$  3 g, respectively; *P* < 0.001) by week 3. Daily food intake was identical between HiPro and CON (see Materials and Methods), but HiPro daily urine volume was higher at week 3 (44.0  $\pm$  5 versus 15  $\pm$  2 ml, respectively; *P* < 0.001). Left kidney weights in HiPro and CON were similar at week 1 (1.011  $\pm$  0.019 versus 0.988  $\pm$  0.022 g, respectively; *P* = 0.44) but were higher in HiPro at week 3 (1.251  $\pm$  0.028 versus 1.048  $\pm$  0.025 g, respectively; *P* < 0.001). In addition, length of accessible distal tubule was similar in HiPro and CON at week 1 (1.041  $\pm$  0.032 versus 0.978  $\pm$  0.030 mm, respectively; *P* = 0.17) but was greater in HiPro at week 3 (1.289  $\pm$  0.041 versus 1.023  $\pm$  0.033 mm, respectively; *P* < 0.001). Bosentan did not affect animal or kidney weight, tubule length, food intake, or urine output in either group.

### Effect of HiPro on Arterial Acid-Base Parameters of Conscious Animals

Table 2 shows that weeks 1 and 3 arterial pH and PCO<sub>2</sub> by blood gases with calculated [HCO<sub>3</sub>] were not different, but plasma TCO<sub>2</sub> by ultrafluorometry was lower in HiPro than CON. Mean BP was not different in HiPro and CON (111.5  $\pm$  2.4 versus 110.1  $\pm$  2.2 mmHg; *P* = 0.67) at week 3.

### Effect of HiPro on Renal Acidification

Figure 1 shows higher urine NAE in HiPro than CON at weeks 1, 2, and 3 (7067  $\pm$  937 versus 4460  $\pm$  639  $\mu$ M/d; *P* <

Table 2. Plasma acid-base data in conscious animals after three weeks of dietary protein (HiPro)<sup>a</sup>

	pH	PCO <sub>2</sub> (mmHg)	Calculated [HCO <sub>3</sub> ] (mM)	Measured [TCO <sub>2</sub> ] (mM)
CON (20% protein) week 1 ( <i>n</i> = 8)	7.42 $\pm$ 0.02	39.9 $\pm$ 1.0	24.0 $\pm$ 0.8	25.0 $\pm$ 0.5
HiPro (50% protein) week 1 ( <i>n</i> = 8)	7.37 $\pm$ 0.02	39.1 $\pm$ 1.0	22.0 $\pm$ 0.7	23.1 <sup>b</sup> $\pm$ 0.6
CON (20% protein) week 3 ( <i>n</i> = 8)	7.41 $\pm$ 0.02	39.2 $\pm$ 1.0	24.1 $\pm$ 0.9	25.2 $\pm$ 0.6
HiPro (50% protein) week 3 ( <i>n</i> = 8)	7.38 $\pm$ 0.02	38.5 $\pm$ 1.0	22.1 $\pm$ 0.8	23.3 <sup>b</sup> $\pm$ 0.5

<sup>a</sup> Values are means  $\pm$  SEM.

<sup>b</sup> *P* < 0.05 versus 20% protein.



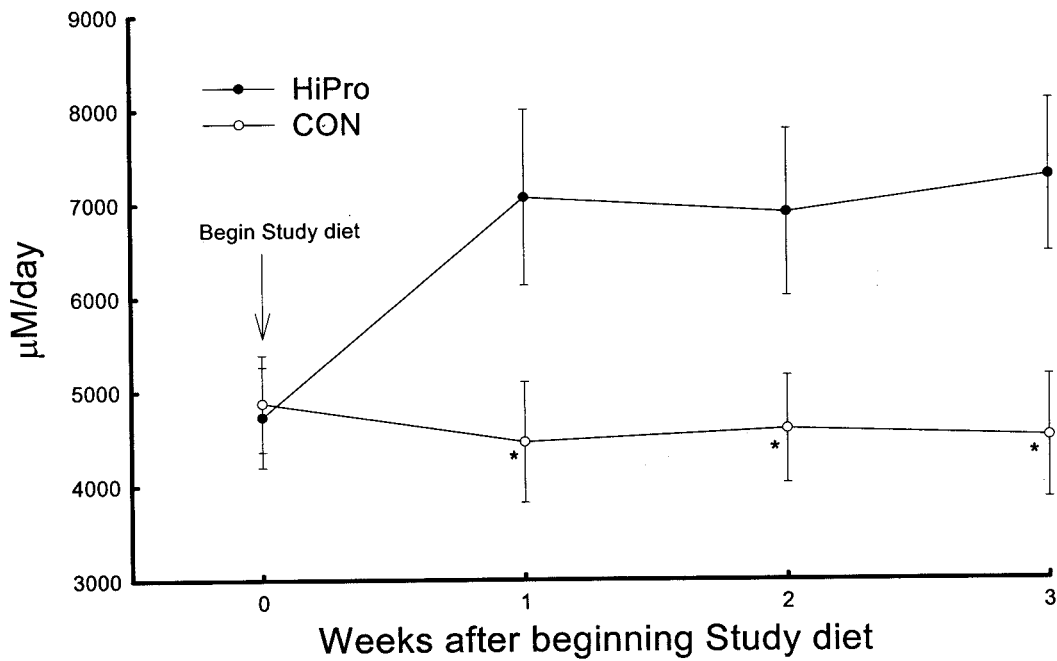


Figure 1. Daily urine net acid excretion (NAE) measured in weekly intervals in conscious rats that initially ate standard chow with 23% protein before being changed to the study diets with 50% (HiPro) and 20% (CON) protein provided as purified casein. \**P* < 0.05 versus CON.

0.04 at week 3). Higher NAE in HiPro was due to higher ammonium (NH<sub>4</sub><sup>+</sup>) excretion (5117 ± 613 versus 2455 ± 353 μM/d; *P* < 0.003) and lower HCO<sub>3</sub> excretion (56 ± 21 versus 257 ± 68 μM/d; *P* < 0.002), but titratable acid excretion was not different between HiPro and CON (2006 ± 338 versus 2261 ± 345 μM/d respectively; NS). Distal nephron net HCO<sub>3</sub> reabsorption was higher at week 1 in HiPro than CON whether

perfused at 6 nl/min (26.1 ± 2.2 versus 12.2 ± 1.4 pmol/mm per min; *P* < 0.001) or 9 nl/min (39.0 ± 3.6 versus 18.2 ± 1.6 pmol/mm per min; *P* < 0.001) as shown in Figure 2. Higher distal nephron net HCO<sub>3</sub> reabsorption in HiPro than CON at week 1 was due to higher H<sup>+</sup> secretion (29.1 ± 2.7 versus 21.6 ± 2.0 pmol/mm per min, *P* < 0.02 for 6 nl/min; 43.5 ± 3.9 versus 26.2 ± 2.5 pmol/mm per min, *P* < 0.002 for 9 nl/min) and less so to lower HCO<sub>3</sub> secretion (−3.0 ± 0.5 versus −6.1 ± 0.8 pmol/mm per min, *P* < 0.001 for 6 nl/min; −4.5 ± 0.6 versus −8.0 ± 1.0 pmol/mm per min, *P* < 0.001 for 9 nl/min). Similarly, Figure 2 shows that distal nephron net HCO<sub>3</sub> reabsorption was higher at week 3 in HiPro than CON whether perfused at 6 nl/min (25.8 ± 2.2 versus 14.4 ± 2.0 pmol/mm per min; *P* < 0.004) or 9 nl/min (37.8 ± 3.2 versus 16.6 ± 1.5 pmol/mm per min; *P* < 0.001) as shown in Figure 2. Higher distal nephron net HCO<sub>3</sub> reabsorption in HiPro than CON at week 3 was due to higher H<sup>+</sup> secretion (28.7 ± 2.8 versus 19.9 ± 1.9 pmol/mm per min, *P* < 0.03 for 6 nl/min; 41.3 ± 4.0 versus 23.0 ± 2.1 pmol/mm per min, *P* < 0.002 for 9 nl/min) and less so to lower HCO<sub>3</sub> secretion (−2.8 ± 0.3 versus −5.5 ± 0.5 pmol/mm per min, *P* < 0.001 for 6 nl/min; −3.5 ± 0.4 versus −6.4 ± 0.8 pmol/mm per min, *P* < 0.001 for 9 nl/min).

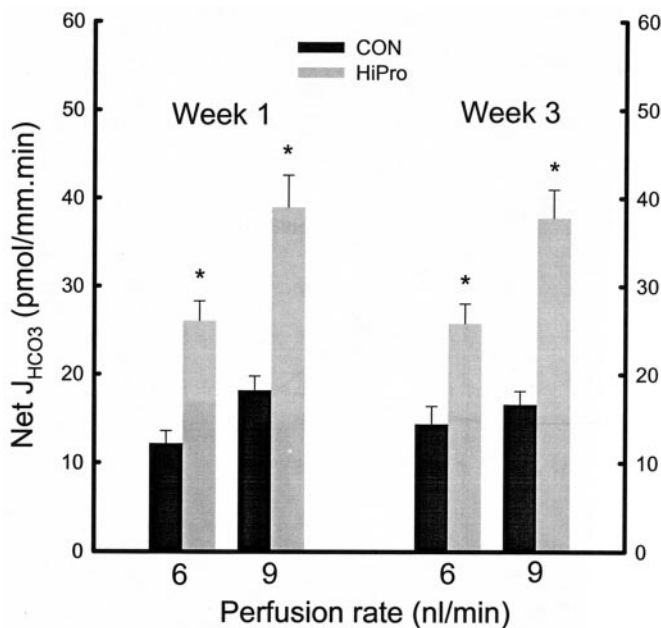


Figure 2. Distal nephron net HCO<sub>3</sub> reabsorption (Net J<sub>HCO<sub>3</sub></sub>) in tubules that were microperfused *in vivo* at 6 or 9 nl/min in HiPro and CON at weeks 1 and 3 of the protocol. \**P* < 0.05 versus CON.

#### Transport Mediators of HiPro-Induced Enhanced Distal Nephron Acidification

Figure 3 shows that at week 1, the net decrease in distal nephron H<sup>+</sup> secretion was greater in HiPro than CON with EIPA (−19.3 ± 1.5 versus −10.9 ± 0.7 pmol/mm per min, *P* < 0.006 at 6 nl/min; −18.9 ± 1.6 versus −11.9 ± 0.9 pmol/mm per min, *P* < 0.006 at 9 nl/min) and bafilomycin (−16.4 ± 1.3 versus −7.6 ± 0.6 pmol/mm per min, *P* < 0.001

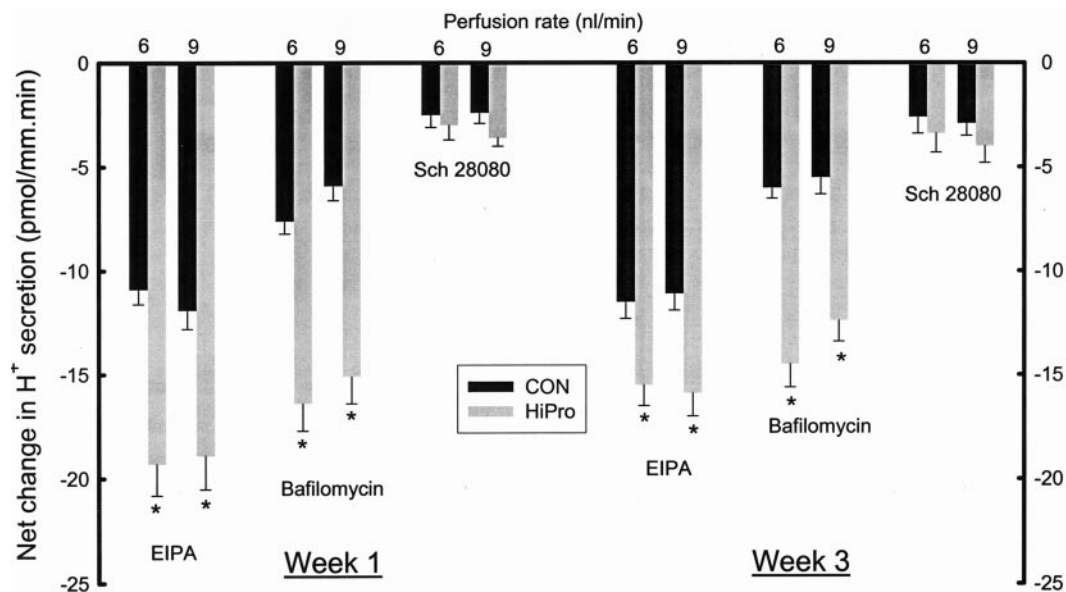


Figure 3. Net change in distal nephron proton ( $H^+$ ) secretion at weeks 1 and 3 in response to *in vivo* microperfusion at 6 or 9 nl/min with inhibitors of  $Na^+/H^+$  exchange (EIPA),  $H^+$ -ATPase (Bafilomycin), and  $H^+,K^+$ -ATPase (Sch 28080). \* $P < 0.05$  versus CON.

at 6 nl/min;  $-15.1 \pm 1.3$  versus  $-5.9 \pm 0.7$  pmol/mm per min,  $P < 0.006$  at 9 nl/min), consistent with enhanced  $Na^+/H^+$  exchange and  $H^+$ -ATPase activity, respectively. Net decrease in  $H^+$  secretion induced by Sch 28080 was not different in HiPro and CON ( $-3.0 \pm 0.7$  versus  $-2.5 \pm 0.6$  pmol/mm per min,  $P = 0.60$  at 6 nl/min;  $-3.6 \pm 0.7$  versus  $-2.4 \pm 0.5$  pmol/mm per min,  $P = 0.18$  for 9 nl/min), consistent with no increased  $H^+,K^+$ -ATPase activity in HiPro. Similarly, net decrease in distal nephron  $H^+$  secretion was greater in HiPro than CON at week 3 with EIPA ( $-15.5 \pm 1.0$  versus  $-11.3 \pm 0.8$  pmol/mm per min,  $P < 0.006$  at 6 nl/min;  $-15.9 \pm 1.1$  versus  $-11.1 \pm 0.8$  pmol/mm per min,  $P < 0.004$  at 9 nl/min) and bafilomycin ( $-14.5 \pm 1.1$  versus  $-6.0 \pm 0.5$  pmol/mm per min,  $P < 0.001$  at 6 nl/min;  $-12.4 \pm 1.0$  versus  $-5.5 \pm 0.8$  pmol/mm per min,  $P < 0.001$  at 9 nl/min), consistent with enhanced  $Na^+/H^+$  exchange and  $H^+$ -ATPase activity, respectively, as shown in Figure 3. Net decrease in  $H^+$  secretion induced by Sch 28080 was not different in HiPro and CON ( $-3.4 \pm 0.9$  versus  $-2.6 \pm 0.8$  pmol/mm per min,  $P = 0.52$  at 6 nl/min;  $-4.0 \pm 0.8$  versus  $-2.9 \pm 0.6$  pmol/mm per min,  $P = 0.29$  for 9 nl/min), consistent with no increased activity of  $H^+,K^+$ -ATPase activity in HiPro.

#### Effect of HiPro on Renal ET-1 Production

Figure 4 shows that HiPro compared with CON had similar urine ET-1 excretion at week 0 ( $42.9 \pm 5.8$  versus  $33.2 \pm 3.7$  fmol/kg body wt per d;  $P = 0.18$ ), but HiPro was higher at week 1 ( $122.4 \pm 26.8$  versus  $39.5 \pm 3.9$  fmol/kg body wt per d;  $P < 0.009$ ), week 2 ( $89.6 \pm 16.1$  versus  $30.8 \pm 3.4$  fmol/kg body wt per d;  $P < 0.004$ ), and week 3 ( $80.0 \pm 15.7$  versus  $29.0 \pm 3.9$  fmol/kg body wt per d;  $P < 0.008$ ). In addition, Figure 4 shows that HiPro and CON had similar ET-1 addition to microdialysate at week 0 ( $275.2 \pm 81.0$  versus  $249.3 \pm 30.5$  fmol/g kidney wt/ per min;  $P = 0.77$ ), but HiPro had greater

renal cortical microdialysate addition at week 1 ( $612.4 \pm 81.0$  versus  $255.2 \pm 32.5$  fmol/g kidney wt per min;  $P < 0.002$ ), week 2 ( $456.8 \pm 62.5$  versus  $216.3 \pm 34.1$  fmol/g kidney wt per min;  $P < 0.005$ ), and week 3 ( $386.1 \pm 49.4$  versus  $230.8 \pm 29.2$  fmol/g kidney wt per min;  $P < 0.02$ ). In addition, Figure 5 shows a qualitative increase in renal cortical mRNA in HiPro than CON at week 3. HiPro had higher urine flow than CON, and high urine flow might itself increase urine ET-1 excretion (18). Consequently, we studied CON ingesting 20% protein diet and distilled  $H_2O$  compared with those ingesting 4% dextrose-containing drinking water (CON-D4W) to increase daily urine volume to a level comparable to that of HiPro without providing additional dietary protein. At week 3, CON-D4W compared with CON had higher daily urine volume ( $40.2 \pm 3$  versus  $14 \pm 1$  ml/d, respectively;  $n = 8$ ;  $P < 0.001$ ) and numerically higher urine ET-1 excretion ( $43.6 \pm 9.5$  fmol/kg body wt per d versus respective week 3 CON;  $n = 8$ ;  $P = 0.18$ ). Nevertheless, ET-1 addition to renal cortical microdialysate in animals when compared with those without the dextrose-containing drinking solution was not different ( $238 \pm 39$  fmol/g kidney wt per min versus respective week 3 CON;  $n = 8$ ;  $P = 0.88$ ). Although qualitative comparison of CON-D4W and CON ET-1 mRNA expression suggested a small difference (Figure 5), absolute quantitative analysis failed to show any differences (data not shown).

#### Effect of ET-1 Receptor Blockade on Arterial Blood and Urine Parameters

Table 3 shows that bosentan did not affect CON arterial plasma acid-base parameters at weeks 1 or 3. By contrast, Table 4 shows that HiPro receiving bosentan had lower plasma  $TCO_2$  at week 1 but not week 3. In addition, mean BP was not different in bosentan-ingesting compared with noningesting HiPro ( $109.7 \pm 2.3$  versus  $111.5 \pm 2.4$  mmHg;  $P = 0.60$ ) or

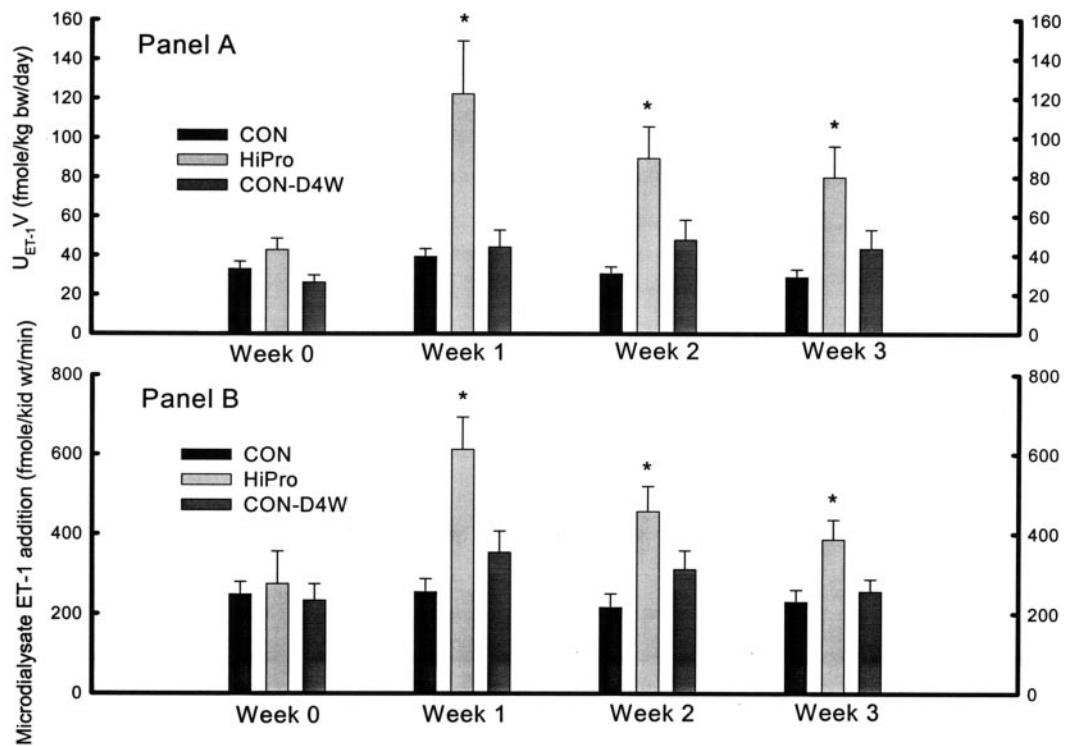


Figure 4. Daily urine endothelin-1 excretion ( $U_{ET-1}V$ ; A) and ET-1 microdialysate addition (B) at weekly intervals in HiPro and CON conscious rats. \* $P < 0.05$  versus CON.

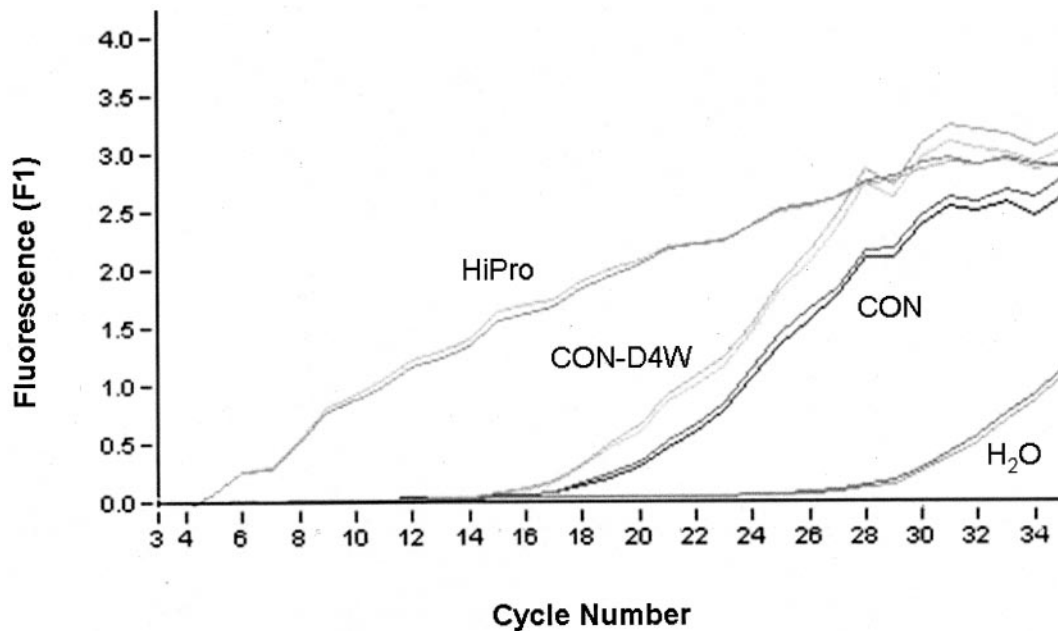


Figure 5. Qualitative comparison of ET-1 mRNA using real-time PCR and LightCycler technology. CON indicates animals ingesting the 20% experimental diet and CON-D4W indicates CON drinking 4% dextrose solution to increase urine flow to that comparable to HiPro. Earlier amplification indicates greater number of ET-1 mRNA copies.

CON ( $110.4 \pm 2.3$  versus  $110.1 \pm 2.2$  mmHg;  $P = 0.93$ ). Figure 6 shows that HiPro receiving bosentan had lower urine NAE ( $5704 \pm 594$  versus  $7067 \pm 937$   $\mu\text{M}/\text{d}$ ;  $P < 0.05$ , paired  $t$ ) at week 1, but NAE was comparable without and with

bosentan at week 3. Lower NAE at week 1 in HiPro with bosentan was due to lower  $\text{NH}_4^+$  excretion ( $3715 \pm 416$  versus  $5117 \pm 613$   $\mu\text{M}/\text{d}$ ;  $P < 0.03$ , paired  $t$ ) and higher  $\text{HCO}_3^-$  excretion ( $231 \pm 42$  versus  $56 \pm 21$   $\mu\text{M}/\text{d}$ ;  $P < 0.001$ , paired

Table 3. Plasma acid-base parameters of control animals in response to bosentan<sup>a</sup>

	pH	PCO <sub>2</sub> (mmHg)	Calculated [HCO <sub>3</sub> ] (mM)	Measured [TCO <sub>2</sub> ] (mM)
20% protein (control) week 1 ( <i>n</i> = 8)	7.41 ± 0.02	39.2 ± 1.0	24.1 ± 0.9	25.2 ± 0.6
20% protein (+bosentan) week 1 ( <i>n</i> = 8)	7.40 ± 0.02	38.9 ± 0.9	23.7 ± 0.6	24.8 ± 0.6
20% protein (+bosentan) week 3 ( <i>n</i> = 8)	7.39 ± 0.02	39.8 ± 1.0	23.6 ± 0.7	24.5 ± 0.5
20% protein (+bosentan) week 3 ( <i>n</i> = 8)	7.40 ± 0.02	38.2 ± 0.9	23.1 ± 0.6	24.1 ± 0.5

<sup>a</sup> Values are means ± SEM.

Table 4. Plasma acid-base parameters of HiPro animals in response to bosentan<sup>a</sup>

	pH	PCO <sub>2</sub> (mmHg)	Calculated [HCO <sub>3</sub> ] (mM)	Measured [TCO <sub>2</sub> ] (mM)
50% protein (HiPro) week 1 ( <i>n</i> = 8)	7.37 ± 0.02	39.1 ± 1.0	22.0 ± 0.7	23.1 ± 0.6
50% protein (+bosentan) week 1 ( <i>n</i> = 8)	7.34 ± 0.02	36.1 ± 1.1	19.0 ± 0.9	19.9 <sup>b</sup> ± 0.5
50% protein (+bosentan) week 3 ( <i>n</i> = 8)	7.38 ± 0.02	38.3 ± 1.0	22.2 ± 0.6	23.0 ± 0.5
50% protein (+bosentan) week 3 ( <i>n</i> = 8)	7.37 ± 0.02	37.6 ± 1.0	21.3 ± 0.6	22.2 ± 0.5

<sup>a</sup> Values are means ± SEM.

<sup>b</sup> *P* < 0.05 versus HiPro.

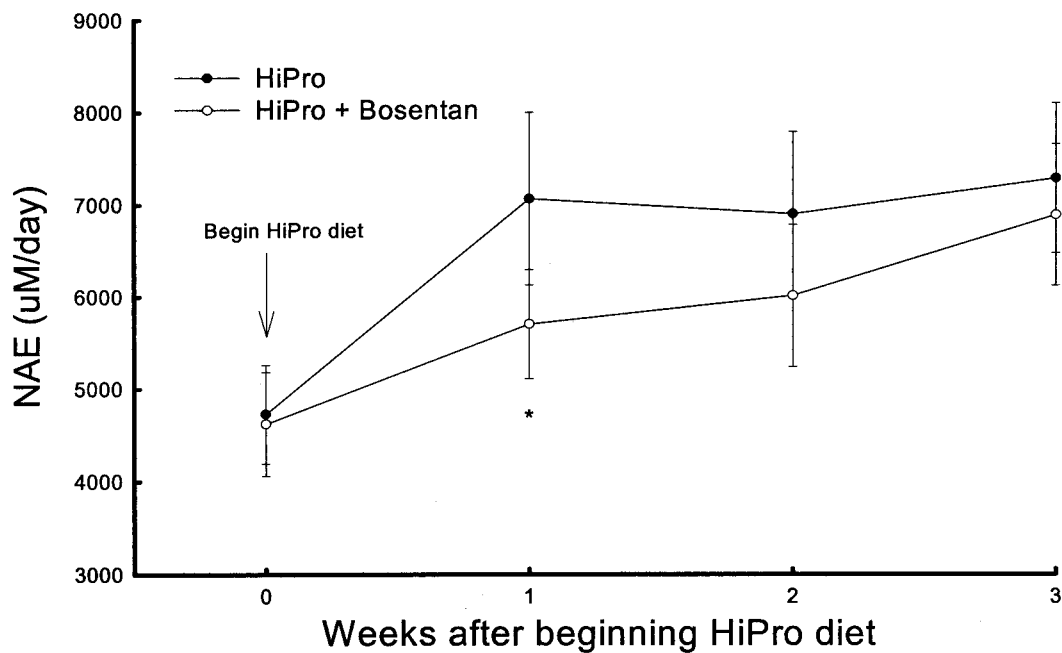


Figure 6. Daily urine NAE in HiPro animals that did and did not ingest the ET A/B receptor antagonist bosentan. \**P* < 0.05 versus CON.

*t*), but titratable acid excretion was not different (2006 ± 338 versus 2221 ± 304 μM/d, respectively; *P* = 0.64).

#### Effect of ET-1 Receptor Blockade on HiPro-Induced Changes in Distal Nephron Acidification

Distal nephron acidification was not different between CON receiving and not receiving bosentan (data not shown). At week 1, Figure 7 shows that HiPro receiving bosentan had lower distal tubule net HCO<sub>3</sub> reabsorption when perfused at 9 nl/min (21.5 ± 2.1 versus 39.0 ± 3.6 pmol/mm

per min; *P* < 0.001). Lower net HCO<sub>3</sub> reabsorption at week 1 was due to lower H<sup>+</sup> secretion (28.7 ± 2.5 versus 43.5 ± 3.9 pmol/mm per min; *P* < 0.007) and higher HCO<sub>3</sub> secretion (−6.1 ± 0.8 versus −3.0 ± 0.5 pmol/mm per min; *P* < 0.006). Figure 7 also shows that week 3 HiPro animals that received bosentan and perfused at 9 nl/min had lower distal tubule net HCO<sub>3</sub> reabsorption (22.4 ± 1.9 versus 37.8 ± 3.2 pmol/mm per min; *P* < 0.001). This lower distal tubule net HCO<sub>3</sub> reabsorption was due to lower H<sup>+</sup> secretion (28.4 ± 2.4 versus 41.3 ± 4.0 pmol/mm per min; *P* < 0.016) and

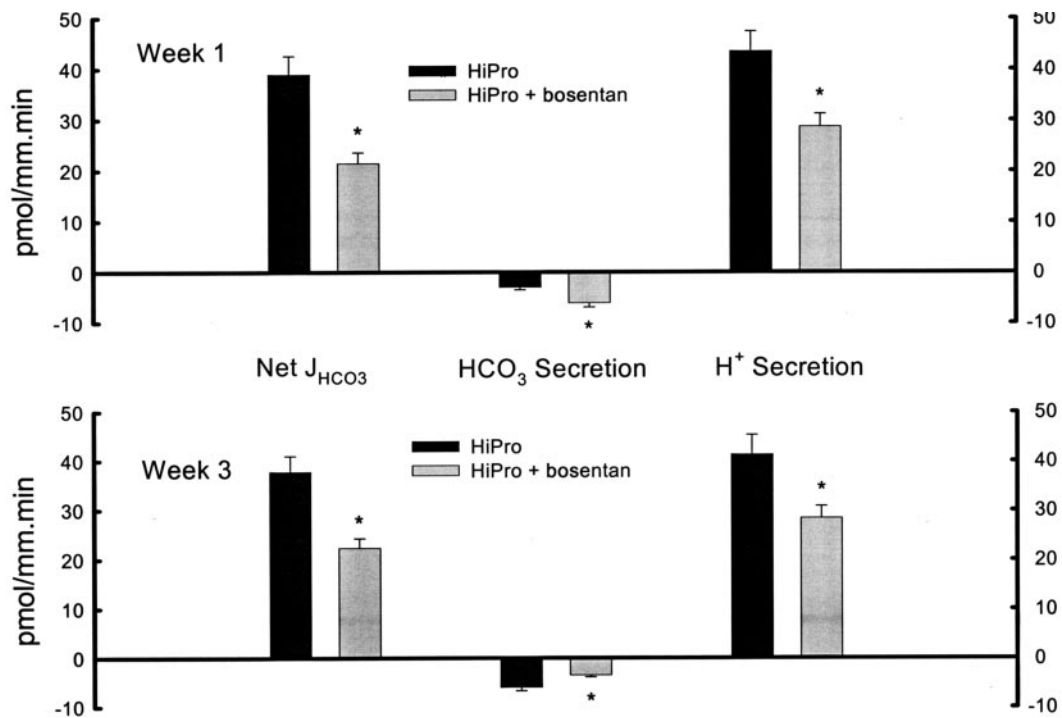


Figure 7. Distal tubule net  $\text{HCO}_3^-$  reabsorption ( $\text{Net } J_{\text{HCO}_3}$ ) and its components,  $\text{HCO}_3^-$  and  $\text{H}^+$  secretion, at weeks 1 and 3 by *in vivo* microperfusion in HiPro animals that did and did not ingest the ET A/B receptor antagonist bosentan. \* $P < 0.05$  versus CON.

higher  $\text{HCO}_3^-$  secretion ( $-6.0 \pm 0.7$  versus  $-3.5 \pm 0.4$  pmol/mm per min;  $P < 0.006$ ).

#### Effect of ET-1 Receptor Blockade on Enhanced $\text{H}^+$ Transporter Activity Induced by HiPro

At week 1, net decrease in distal nephron  $\text{H}^+$  secretion was not different in bosentan-ingesting compared with noningesting HiPro perfused at 9 nl/min with EIPA ( $-16.4 \pm 1.8$  versus  $-18.9 \pm 1.6$  pmol/mm per min;  $P = 0.32$ ) and bafilomycin ( $-14.0 \pm 1.5$  versus  $-15.1 \pm 1.3$  pmol/mm per min;  $P = 0.59$ ), consistent with no additional effect of these  $\text{H}^+$  inhibitors on  $\text{Na}^+/\text{H}^+$  exchange and  $\text{H}^+$ -ATPase activity, respectively, in HiPro with ET A/B receptor blockade (Figure 8). There was no difference in net  $\text{H}^+$  secretion decrease in HiPro perfused with Sch 28080 ( $-4.4 \pm 0.6$  versus  $-3.0 \pm 0.7$  pmol/mm per min;  $P = 0.15$ ), consistent with no additional effect of ET A/B receptor blockade on  $\text{H}^+$ , $\text{K}^+$ -ATPase activity in HiPro. Similarly, net decrease in distal nephron  $\text{H}^+$  secretion at week 3 was not different in the bosentan-ingesting compared with the noningesting HiPro animals perfused at 9 nl/min with EIPA ( $-13.4 \pm 1.2$  versus  $-15.9 \pm 1.1$  pmol/mm per min;  $P = 0.15$ ) and bafilomycin ( $-11.0 \pm 1.0$  versus  $-12.4 \pm 1.0$  pmol/mm per min;  $P = 0.34$ ), consistent with no additional effect of these  $\text{H}^+$  inhibitors on  $\text{Na}^+/\text{H}^+$  exchange and  $\text{H}^+$ -ATPase activity, respectively, in HiPro with ET A/B receptor blockade. There was also no difference in net  $\text{H}^+$  secretion decrease in HiPro perfused with Sch 28080 ( $-5.0 \pm 0.8$  versus  $-3.4 \pm 0.9$  pmol/mm per min;  $P = 0.21$ ), consistent with no additional effect of ET A/B receptor blockade on  $\text{H}^+$ , $\text{K}^+$ -ATPase activity in HiPro.

## Discussion

The present studies show that increased dietary protein as purified casein augments distal nephron acidification and does so by increasing  $\text{H}^+$  secretion through increased  $\text{Na}^+/\text{H}^+$  exchange and increased  $\text{H}^+$ -ATPase activity and to a lesser extent by decreasing  $\text{HCO}_3^-$  secretion. Increased dietary protein increased renal ET-1 production, and the data support that each component of increased distal nephron acidification was mediated by increased ET activity. That the studies used *in vivo* microperfusion of the distal nephron supports that the observed ET effects were mediated through effects on transport rather than hemodynamics. These studies show that ET is a mediator of increased renal acidification in response to dietary protein, the common acid challenge faced by humans.

ET increases  $\text{Na}^+/\text{H}^+$  exchange in renal epithelia *in vitro* (19,20), and ET A/B receptor antagonism inhibits  $\text{Na}^+/\text{H}^+$  exchange in the distal nephron *in vivo* (14), but we are not aware of studies showing that ET increases  $\text{H}^+$ -ATPase activity. This suggests that the increased  $\text{H}^+$ -ATPase activity that is reduced by ET A/B receptor blockade is an indirect effect of ET, possibly acting through another agent. A possible scenario is that increased renal ET production induced by dietary protein increases adrenal secretion of aldosterone (21) that in turn increases distal nephron  $\text{H}^+$ -ATPase activity (22). Further studies will be necessary to explore this hypothesis.

Table 2 shows lower plasma  $\text{TCO}_2$  in HiPro compared with CON, consistent with a relative metabolic acidosis in HiPro compared with CON. Because plasma  $\text{TCO}_2$  remained at this slightly reduced level at weeks 1 and 3, it seems that increased



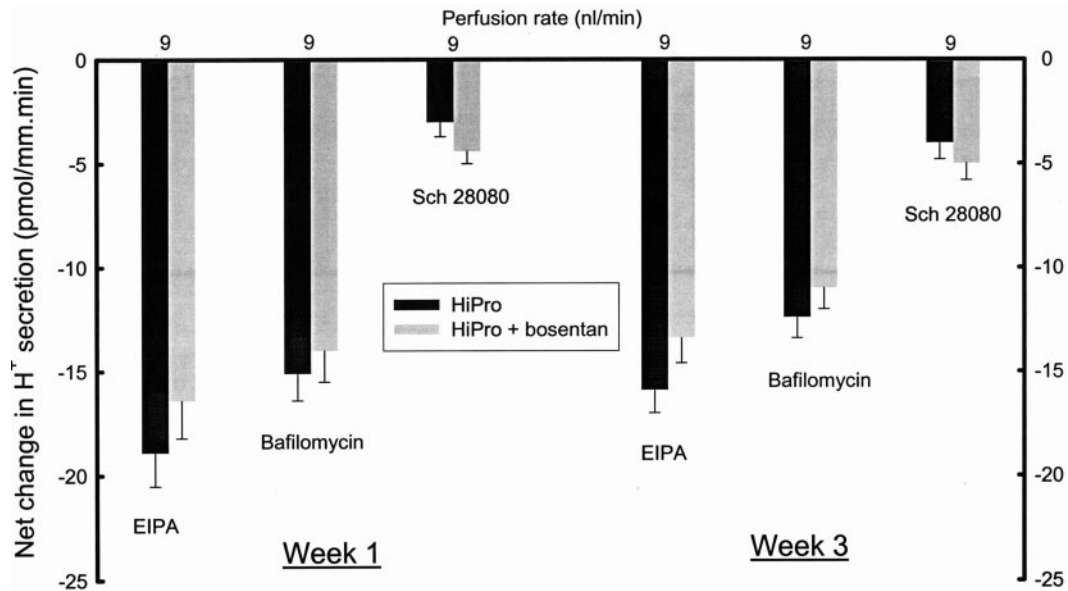


Figure 8. Net change in distal nephron proton ( $H^+$ ) secretion in HiPro at weeks 1 and 3 in response to *in vivo* microperfusion at 6 or 9 nl/min with inhibitors of  $Na^+/H^+$  exchange (EIPA),  $H^+$ -ATPase (Bafilomycin), and  $H^+,K^+$ -ATPase (Sch 28080). \* $P < 0.05$  versus CON.

dietary protein as casein leads to a steady-state but not progressive acidosis. That the metabolic acidosis was not progressive is likely due to the marked increase in urine NAE (Figure 1). Dietary mineral acid causes mild net acid retention that mediates the sustained associated increase in urine NAE (23). Also, increased  $[H^+]$  *in vitro* increases ET-1 release from renal microvascular endothelium (24) and renal epithelium (20), and so increased endogenous ET might contribute to the untoward effects postulated for chronic metabolic acidosis (25).

Figure 1 shows that bosentan decreased urine NAE in HiPro at week 1 but not at week 3, suggesting greater ET dependence of HiPro-induced acidification at week 1. Although the net reduction of distal nephron acidification measured per millimeter of tubule length was not different at weeks 1 and 3, the perfused distal nephron segment was longer at week 3, consistent with tubule hypertrophy induced by HiPro. Because there was residual acidification in animals that ingested bosentan, the longer tubule at week 3 might have allowed for more overall ET-independent acidification at this time point.

In summary, increased dietary protein augments distal nephron acidification through an ET-dependent mechanism. The data support that ET contribute to the overall acidification response to this common dietary challenge to systemic acid-base homeostasis faced by humans.

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