Endothelin-Induced Increased Nitric Oxide Mediates Augmented Distal Nephron Acidification as a Result of Dietary Protein

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Tested was the hypothesis that enhanced nitric oxide (NO) production that is stimulated by increased renal endothelin activity mediates decreased distal nephron HCO3 secretion that is induced by dietary protein. Munich-Wistar rats that ate minimum electrolyte diets with 50% casein-provided protein (HiPro) compared with controls that ate 20% protein for 3 wk had higher urine excretion of endothelin-1 (80 ± 15.7 versus 29 ± 3.9 fmol/kg body wt per d; P < 0.02) and of the NO metabolites NO2/NO3 (21.2 ± 1.9 versus 14.9 ± 0.8 μmol/kg body wt per d; P < 0.03). Bosentan, an endothelin A/B receptor antagonist, reduced HiPro rats’ urine excretion of net acid (5859 ± 654 versus 8017 ± 1103 μmol/d; P < 0.03, paired t test) and NO2/NO3 (18.1 ± 1.1 versus 22.9 ± 2.0 μmole/kg body wt per d; P < 0.05, paired t test). L-NAME, an NO synthase inhibitor, also decreased urine net acid excretion (6621 ± 717 versus 8449 ± 1086 μmol/d; P < 0.05, paired t test) but was not additive to bosentan. L-NAME increased in situ late distal nephron HCO3 delivery in HiPro rats (18.8 ± 1.7 versus 9.6 ± 1.4 pmol/mm per min; P < 0.001) that was mediated by increased distal nephron HCO3 secretion (−7.2 ± 0.7 versus −3.5 ± 0.4 pmol/mm per min; P < 0.001) without changes in distal nephron transtubule HCO3 permeability or H+ secretion. Bosentan decreased H+ secretion and increased HCO3 secretion in the distal nephron of HiPro rats, but L-NAME had no additive effect on either component. The data support that dietary protein augments distal nephron acidification through decreased HCO3 secretion that is mediated through endothelin-stimulated NO.


Enhanced renal endothelin production that is induced by increased dietary protein augments distal nephron acidification through increased H+ secretion and decreased HCO3 secretion (1,2). Augmented distal nephron H+ secretion in this setting is due to an endothelin-stimulated increase in Na+/H+ exchange (1,2) and to endothelin-induced increased aldosterone secretion that in turn stimulates H+-ATPase activity (2), supporting the important role for endothelin as a mediator of augmented distal nephron H+ secretion induced by increased dietary protein. Although endothelin receptor antagonism inhibits the decrease in distal nephron HCO3 secretion that is induced by dietary protein (1,2) and systemic endothelin infusion reduces distal nephron HCO3 secretion that is induced by dietary HCO3 (3), the mechanism by which endothelin reduces distal nephron HCO3 secretion is not known. In addition to increasing renal production of endothelin, increased dietary protein increases urine excretion of nitric oxide (NO) metabolites (4). Furthermore, endothelin increases renal NO production (5), and NO increases acidification in the proximal (6) and distal (7) nephron. Consequently, we tested the hypothesis that increased renal endothelin production that is induced by increased dietary protein reduces distal nephron HCO3 secretion through increased NO.

Materials and Methods

Animals and Diet Protocol

Male and female Munich-Wistar rats (Harlan Sprague-Dawley, Houston, TX; 200 to 228 g) ate standard rat chow (Prolab RMH 2500 with 23% protein, Purina, Indianapolis, IN) for 1 wk, then ate a custom minimum electrolyte diet with protein as purified high nitrogen casein (ICN Nutritional Biochemicals, Cleveland, OH) for 3 wk and drank distilled H2O ad libitum. Rats that were on the high-protein diet (HiPro) ate custom diet with 50% protein, and controls ate 20%. In preliminary studies, similar-weight rats ate 24.6 ± 0.9 and 27.1 ± 1.2 g/d, respectively (n = 4, P = 0.15), so all rats received 24 g/d diet to ensure similar diet intake and complete ingestion of drug mixed with diet in rats that were given drugs. Some received bosentan (Actelion, Allschwil, Switzerland), a nonpeptide endothelin A/B receptor antagonist (8), mixed with study diet at 100 mg/kg body wt per d. This oral dose blocks action of pressor doses of intravenous big ET-1 for >24 h (8). Preliminary studies determined that the minimal chronic dose of the NO synthase (NOS) inhibitor N-nitro-l-arginine methyl ester (L-NAME) that reduced urine net acid excretion (NAE) in paired HiPro rats (6451 ± 785 versus 8488 ± 904 μmol/d; n = 4 each; P < 0.03, paired t) was 25 mg/kg body wt per d L-NAME and was the dose used in these studies.

Urine NAE in Conscious Animals

Daily urine NAE was measured (9) in a 24-h sample that was collected on protocol day 28 in eight each of HiPro rats and controls in...
metabolic cages. The effect of endothelin receptor blockade and of NOS inhibition on urine NAE was examined in paired and separate groups of eight each (four not ingesting and four ingesting) of HiPro rats and control.

**Arterial Total CO₂ in Conscious Animals**

Arterial plasma total CO₂ (TCO₂) from a chronic carotid arterial catheter in eight each of awake, gently restrained, and calm HiPro rats and controls was measured by ultrafluorometry (see below) to assess the effect of 3 wk of HiPro on this acid-base parameter.

**GFR in Conscious Animals**

GFR was measured in eight each of awake, gently restrained, and calm HiPro rats and controls with standard clearance techniques using \(^3\)H-inulin as described previously in anesthetized animals (10). The effect of endothelin receptor blockade and of NOS inhibition on GFR was examined in paired and separate groups of eight each (four not ingesting and four ingesting inhibitor) of HiPro rats and controls.

**Micropuncture Protocol**

Accessible rat distal nephron segments underwent paired free-flow fluid collections from late then early portions (9) or underwent in vivo micropuncture (11). *In situ* early distal flow rate for HiPro rats and controls was 9.4 ± 0.7 \((n = 6)\) and 6.4 ± 0.4 nl/min \((n = 8)\), respectively. Consequently, distal nephrons of both HiPro rats and controls were perfused at 6 and 9 nl/min with a Hampel pump. When comparisons were done within HiPro rats or controls, only the 9- or 6-nl/min flow rate was used. Distal nephron transepithelial potential difference was measured to calculate blood-to-lumen HCO₃ permeability (11). Tubule segment length was determined using an injected latex cast (11). Stellate vessel plasma [HCO₃] was measured to determine peritubular blood-to-lumen HCO₃ gradient for calculating transepithelial H⁺/HCO₃ passive permeability (11). Diet but not H₂O was withheld the evening before micropuncture to yield higher baseline HCO₃ reabsorption (12), as done previously (11).

Perfusion solutions are in Table 1. Standard perfusate (solution 1) contained 5 mM HCO₃ to approximate early distal tubule \([\text{HCO}_3^-]\) *in situ* (11). Solution 2 was HCO₃⁻- and chloride (Cl⁻)-free and contained acetazolamide to inhibit transtubule H⁺/HCO₃ transport and thereby determine passive blood-to-lumen H⁺/HCO₃ permeability (11) that was used to calculate passive blood-to-lumen HCO₃ secretion when perfusing with the HCO₃-containing solution 1 (11). We also measured Cl⁻-dependent luminal HCO₃ accumulation with solution 3 having Cl⁻ but no HCO₃ to allow Cl⁻-dependent HCO₃ secretion (11). Solution 3 also permitted determination of “apparent” blood-to-lumen HCO₃ permeability to calculate distal nephron H⁺/HCO₃ secretion when perfusing with HCO₃-containing solution 1 (11). The latter value is "total" HCO₃ secretion whereas "net" HCO₃ secretion is total HCO₃ secretion minus the passive component (calculated with permeability determined with solution 3). All perfusing solutions contained raffinose to minimize fluid transport and gluconate substituted for Cl⁻ when necessary (11).

**Calculations**

Urine NAE was the mean for each animal of a group on day 28. Net HCO₃ reabsorption in microperfusion studies was transport during perfusions with the HCO₃-containing solution 1, recognizing that tubule transport is bidirectional (11,12). Luminal HCO₃ accumulation was HCO₃ appearance (collected – initial) for initially HCO₃-free perfusions. Bicarbonate secretion was calculated using blood-to-lumen HCO₃ transport for distal nephron perfusions with the HCO₃-containing solution 1. “Passive” and “apparent” blood-to-lumen HCO₃ permeability were calculated as above to yield “total” secretion (11) that is reported in these studies. Distal nephron H⁺ secretion was calculated for perfusions with solution 1 by subtracting calculated total secretion (a negative value) from measured net HCO₃ reabsorption (11).

**Statistical Analyses**

Immediately after experiment termination, initial and collected perfusate, as well as stellate vessel plasma samples, were analyzed for inulin (11) and for TCO₂ using flow-through ultrafluorometry (13) as done previously (14). All tubule fluid and plasma TCO₂ were measured on the experimental day by comparing sample fluorescence (corrected for H₂O blank) to a standard curve (14). This technique actually measures TCO₂ but we refer to this measured value as HCO₃ for simplicity.

**[ET-1]** in urine was measured using a RIA kit (Peninsula Laboratories, Belmont, CA) after disposable column extraction (Sep-Pak C18, Milford, MA) preconditioned with methanol, H₂O, and acetic acid as described (15) and as done previously (16).

Urine [NO] was measured as described (17). Briefly, urine samples first were centrifuged to remove all suspended and undissolved particles and deproteinized by ethanol precipitation. The samples were diluted 1:20 before measurement. NO was assayed by measuring NOₓ and NO₂ stable end products of NO, by an NO analyzer (Sievers Nitric Oxide Analyzer; Ionics Instruments, Boulder, CO) that uses chemiluminescence. Diluted samples (10 μl) were injected into a purge vessel that contained vanadium that converted NO₃ and NO₂ to NO. NO in the vessel then was propelled by nitrogen into a reaction chamber in which NO was oxidized to NO₂ by oxygen. Chemiluminescence associated with this reaction was displayed in millivolts on the NO analyzer. The signal associated with such a reaction was acquisition and analyzed by NO analysis software that digitizes the data to yield the amount of NO in the sample in micromoles. Every sample was measured in triplicate, and the average of the three readings was taken as the representative of NO content in the sample. The NO activity of the diluting nano-pure water was subtracted from each sample to obtain the true NO content of the sample.

Data were expressed as means ± SEM. One to two distal nephron segments were perfused per animal. When two tubules were perfused, results were averaged to yield a single animal value. Paired perfusates of the same tubule were compared using paired \( t \) test; otherwise, ANOVA was used for multiple group comparisons. Bonferroni method was used for multiple comparisons \((P < 0.05)\) of the same parameter among groups that contained eight each of HiPro rats and controls unless otherwise stated.

**Results**

Effect of HiPro on Urine Flow and GFR in Conscious Rats

Although daily food intake was identical between HiPro rats and controls, HiPro rats had higher urine flow (40 ± 4 versus...
Effect of HiPro on Plasma TCO₂ and Urine NAE of Conscious Rats

HiPro rats had lower plasma TCO₂ by ultrafluorometry (23.0 ± 0.7 versus 25.2 ± 0.6 mM; P < 0.04) and higher urine NAE (8254 ± 1047 versus 4293 ± 476 μmol/d; P < 0.004) than controls. Table 2 shows that higher NAE in HiPro rats was mediated by higher $U_{\text{NH}_4}$,V ($P < 0.001$) and lower $U_{\text{HCO}_3}$V ($P > 0.001$), but $UTAV$ was similar ($P = 0.14$).

HiPro Effect on Distal Nephron Acidification

Table 3 shows that in situ distal nephron net HCO₃ reabsorption was higher in HiPro rats than controls ($P < 0.001$) and was associated with higher early distal HCO₃ delivery in HiPro rats ($P < 0.003$) but with similar fractional reabsorption ($P = 0.94$). The higher early distal nephron HCO₃ delivery in HiPro rats was also associated with higher HCO₃ secretion to the late distal nephron ($P < 0.04$). Whether this higher in situ late distal nephron HCO₃ delivery in HiPro rats was due to differences in distal nephron HCO₃ transport (decreased H⁺ and/or increased HCO₃ secretion) was unclear. These free-flow micropuncture studies were followed by in vivo microperfusion studies to compare distal nephron HCO₃ transport in HiPro rats and controls at identical fluid flows and HCO₃ deliveries. Figure 1 shows higher distal nephron net HCO₃ reabsorption in HiPro rats than controls whether perfused at the 6-μl/min in situ flow rate of controls (25.0 ± 2.3 versus 13.8 ± 1.5 pmol/mm per min; $P < 0.001$) or at the 9-μl/min in situ flow rate of HiPro rats (36.9 ± 3.1 versus 14.1 ± 1.4 pmol/mm per min; $P < 0.001$). Figure 1 also shows that the higher in situ distal nephron net HCO₃ reabsorption in HiPro rats indicated by the free-flow micropuncture studies was due to lower HCO₃ secretion (−3.4 ± 0.3 versus −5.4 ± 0.4 pmol/mm per min, $P < 0.002$ at 6 μl/min; −4.9 ± 0.5 versus −7.7 ± 0.6 pmol/mm per min, $P < 0.003$ at 9 μl/min) and higher H⁺ secretion (28.5 ± 2.6 versus 19.2 ± 1.7 pmol/mm per min, $P < 0.001$ at 6 μl/min; 41.8 ± 3.9 versus 21.8 ± 2.0 pmol/mm per min, $P < 0.001$ at 9 μl/min). These data support that higher HCO₃ delivery to the early distal nephron mediated the increased in situ late distal nephron HCO₃ delivery in HiPro rats.

Effect of HiPro on Urine ET-1 and NO₂/NO₃ Excretion of Conscious Rats

Figure 2 shows that HiPro rats had greater excretion of ET-1 (80 ± 15.7 versus 29 ± 3.9 fmol/kg body wt per d; $P < 0.02$) and of NO₂/NO₃ (21.2 ± 1.9 versus 14.9 ± 0.8 μmol/kg body wt per d; $P < 0.03$) than controls.

Effect of Endothelin A/B Receptor Antagonism and NOS Inhibition on Plasma TCO₂ and Urine NAE of Conscious Rats

Figure 3 shows that plasma TCO₂ of controls that ingested the endothelin A/B receptor antagonist bosentan, the NOS inhibitor L-NAME, or the combination were not different from controls that did not ingest either agent. Figure 3 also shows that plasma TCO₂ of HiPro rats that ingested either bosentan or L-NAME were not different from HiPro rats that did not ingest either agent. By contrast, plasma TCO₂ was lower in HiPro rats that ingested both agents compared with HiPro rats that did not ingest either agent (19.3 ± 0.9 versus 23.0 ± 0.7 μM; $P < 0.024$, ANOVA). Table 2 shows that urine NAE was lower in HiPro rats that ingested inhibitor compared with paired HiPro rats that did not ingest inhibitor with bosentan and L-NAME. Urine NAE was lower in the bosentan-treated HiPro rats because of lower $U_{\text{NH}_4}$,V and higher $U_{\text{HCO}_3}$V, whereas lower urine NAE in the L-NAME–treated HiPro rats was mediated by higher $U_{\text{HCO}_3}$V. When bosentan-treated HiPro rats were compared with the bosentan+L-NAME–treated HiPro rats in a paired manner, L-NAME addition had no additional effect on urine NAE or its components. By contrast, when L-NAME–treated HiPro rats were compared with

Table 2. Urine net acid excretion (NAE) and its components in HiPro rats and controls in response to inhibitors

<table>
<thead>
<tr>
<th></th>
<th>$U_{\text{NH}_4}$,V (μmol/d)</th>
<th>$UTAV$ (μmol/d)</th>
<th>$U_{\text{HCO}_3}$V (μmol/d)</th>
<th>NAE (μmol/d)</th>
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</thead>
<tbody>
<tr>
<td>HiPro</td>
<td>5513 ± 623</td>
<td>2427 ± 310</td>
<td>77 ± 13</td>
<td>8017 ± 1103</td>
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<tr>
<td>HiPro + bosentan</td>
<td>3413 ± 416b</td>
<td>2446 ± 371</td>
<td>407 ± 69b</td>
<td>5859 ± 654b</td>
</tr>
<tr>
<td>HiPro</td>
<td>5819 ± 707</td>
<td>2690 ± 314</td>
<td>60 ± 9.2</td>
<td>8449 ± 1086</td>
</tr>
<tr>
<td>HiPro + L-NAME</td>
<td>4088 ± 572</td>
<td>2888 ± 390</td>
<td>355 ± 42b</td>
<td>6621 ± 717b</td>
</tr>
<tr>
<td>HiPro + bosentan</td>
<td>3715 ± 485</td>
<td>2394 ± 321</td>
<td>265 ± 55</td>
<td>5843 ± 616</td>
</tr>
<tr>
<td>HiPro + L-NAME + L-NAME</td>
<td>3481 ± 416</td>
<td>2785 ± 382</td>
<td>352 ± 70</td>
<td>6004 ± 689</td>
</tr>
<tr>
<td>HiPro + L-NAME</td>
<td>4268 ± 566</td>
<td>2544 ± 312</td>
<td>269 ± 48</td>
<td>6534 ± 703</td>
</tr>
<tr>
<td>HiPro + L-NAME + bosentan</td>
<td>3521 ± 410b</td>
<td>2481 ± 342</td>
<td>377 ± 51</td>
<td>5626 ± 627b</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>2561 ± 351</td>
<td>1830 ± 216</td>
<td>194 ± 22</td>
<td>4198 ± 451</td>
</tr>
<tr>
<td>control + L-NAME</td>
<td>2309 ± 316</td>
<td>1743 ± 219</td>
<td>237 ± 36</td>
<td>3815 ± 442</td>
</tr>
</tbody>
</table>

*Values are means ± SEM; each group had four animals each. L-NAME, N-nitro-l-arginine methyl ester; NH₄⁺, ammonium; TA, titratable acidity; HCO₃⁻, bicarbonate.

*bP < 0.05 versus baseline, paired t test.
Table 3. In situ distal nephron HCO₃ transport of HiPro and control rats

<table>
<thead>
<tr>
<th>HiPro rats</th>
<th>ED [HCO₃] (mM)</th>
<th>LD [HCO₃] (mM)</th>
<th>ED HCO₃ Delivery (pmol/mm per min)</th>
<th>LD HCO₃ Delivery (pmol/mm per min)</th>
<th>Net HCO₃ Reabsorption (pmol/mm per min)</th>
<th>Fractional CO₃ Reabsorption (pmol/mm per min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HiPro</td>
<td>5.7 ± 0.5</td>
<td>3.0 ± 0.5</td>
<td>53.6 ± 4.6</td>
<td>9.6 ± 1.4</td>
<td>44.0 ± 3.0</td>
<td>0.82 ± 0.07</td>
</tr>
<tr>
<td>HiPro+bosentan</td>
<td>7.2 ± 0.6</td>
<td>7.9 ± 0.8 b</td>
<td>64.0 ± 5.1</td>
<td>31.6 ± 2.7 b</td>
<td>32.4 ± 3.0 b</td>
<td>0.51 ± 0.04 b</td>
</tr>
<tr>
<td>HiPro+L-NAME</td>
<td>6.0 ± 0.6</td>
<td>7.5 ± 0.7 b</td>
<td>44.4 ± 3.6</td>
<td>18.8 ± 1.7 b</td>
<td>25.7 ± 2.4 b</td>
<td>0.58 ± 0.05 b</td>
</tr>
<tr>
<td>HiPro+bosentan+L-NAME</td>
<td>7.0 ± 1.0</td>
<td>8.8 ± 0.9 b</td>
<td>47.6 ± 4.9</td>
<td>21.1 ± 2.4 b</td>
<td>26.5 ± 2.4 b</td>
<td>0.55 ± 0.05 b</td>
</tr>
</tbody>
</table>

Controls

| control     | 5.2 ± 0.5      | 3.1 ± 0.4      | 33.3 ± 2.8                        | 5.8 ± 0.8                         | 27.8 ± 2.2                             | 0.84 ± 0.08                                   |
| control+bosentan | 5.5 ± 0.5      | 3.5 ± 0.4      | 33.0 ± 1.1                        | 6.3 ± 0.7                         | 26.7 ± 2.4                             | 0.81 ± 0.08                                   |
| control+L-NAME  | 4.6 ± 0.5      | 4.0 ± 0.4      | 24.0 ± 2.6                        | 6.0 ± 0.7                         | 18.1 ± 2.1                             | 0.76 ± 0.08                                   |
| control+bosentan+L-NAME | 4.5 ± 0.5      | 3.7 ± 0.4      | 22.5 ± 2.7                        | 5.9 ± 0.7                         | 16.7 ± 2.0                             | 0.74 ± 0.08                                   |

Values are means ± SEM. All groups had eight animals each. ED, early distal nephron; LD, late distal nephron.

*P < 0.01 versus HiPro, ANOVA.

![Figure 1](image1.png)  
![Figure 2](image2.png)

**Figure 1.** Distal nephron net HCO₃ reabsorption (Net J₇HCO₃) and its components, HCO₃ and H⁺ secretion, by in vivo microperefusion in rats that were fed a high-protein diet (HiPro) and controls after 3 wk of diet. Positive/negative values indicate reabsorption/secrretion, respectively. *P < 0.05 versus controls.

The L-NAME+bosentan-treated HiPro rats in a paired manner, bosentan addition decreased overall urine NAE by reducing UNH⁺,V. Table 2 shows that urine NAE was not different in controls that ingested the combination of bosentan+L-NAME. Although inhibitor-induced decreased GFR did not contribute to lower urine NAE in bosentan-treated compared with untreated HiPro rats (2054 ± 186 versus 2175 ± 209 µmol/min, respectively; P = 0.29, paired t test), lower GFR might have contributed to reduced urine NAE in L-NAME-treated (1606 ± 144 versus 2296 ± 215 µl/min; P < 0.02, paired t test) and the combination-treated (1451 ± 141 versus 2253 ± 210 µl/min; P < 0.01, paired t test) HiPro rats.

**Effect of Endothelin A/B Receptor Antagonism on Urine NO₂/NO₃ Excretion in Conscious Animals**

Bosentan-treated HiPro rats had lower urine NO₂/NO₃ excretion than paired HiPro rats that did not ingest bosentan (18.1 ± 1.1 versus 22.9 ± 2.0 µmol/kg body wt per d; P < 0.05, paired t test; n = 4 animals each). By contrast, urine NO₂/NO₃ excretion was not different in paired bosentan-treated and untreated controls (12.8 ± 1.0 versus 14.5 ± 1.7 µmol/kg body wt per d; P = 0.31, paired t test; n = 4 animals each).

**Figure 2.** Urine endothelin-1 (ET-1; left) and nitric oxide metabolites (NO₂/NO₃; right) excretion in conscious HiPro rats and controls after 3 wk. *P < 0.05 versus controls.

**Effect of ET-1 Receptor Blockade and NOS Inhibition on HiPro-Induced Increases in Distal Nephron Acidification**

Table 3 shows that bosentan, L-NAME, and their combination increased in situ late distal [HCO₃] and late distal HCO₃ delivery in HiPro rats but not in controls. Table 3 also shows that these inhibitors and their combination reduced distal nephron net and fractional HCO₃ reabsorption in HiPro rats but not in controls. Distal nephron net HCO₃ reabsorption was not different among controls that ingested bosentan, L-NAME, or their combination compared with controls that did not ingest either agent. The inhibitor-induced increases in in situ HCO₃ deliveries to the late distal nephron of HiPro rats were associated with inhibitor-induced differences in HCO₃ deliveries to
the early distal nephron, complicating interpretation of these data from these free-flow micropuncture studies as to the mechanism(s) of these inhibitor-induced increased HCO₃ deliveries to the late distal nephron of HiPro rats. Specifically, these free-flow micropuncture studies did not determine whether the inhibitor-induced increase in HCO₃ deliveries were mediated by inhibitor-induced increases in blood-to-lumen HCO₃ permeability, by decreased H⁺ secretion, and/or by increased HCO₃ secretion. For addressing these mechanistic concerns, these free-flow micropuncture studies were followed by in vivo microperfusion studies in which distal nephrons of HiPro rats and controls were perfused with solutions of identical [HCO₃] and at identical perfusion rates. This technique also allows estimation of transtubule HCO₃ permeability and for calculation of the components of distal nephron net HCO₃ reabsorption, H⁺ and HCO₃ secretion (see Materials and Methods).

Figure 4 shows no difference in peritubular blood-to-lumen passive permeability within HiPro rats and controls when animals that ingested inhibitor were compared with their respective baseline. Furthermore, there was no difference in permeability between HiPro rats and controls at baseline or with either inhibitor or their combination. Figure 5 shows no differences in distal nephron acidification by in vivo microperfusion among controls that ingested either inhibitor or their combination compared with baseline. By contrast, Figure 6 shows that HiPro rats that ingested bosentan had lower distal nephron net HCO₃ reabsorption (22.4 ± 1.9 versus 37.8 ± 3.2 pmol/mm per min; P < 0.001) that was due to greater HCO₃ secretion (−6.0 ± 0.7 versus −3.5 ± 0.4 pmol/mm per min; P < 0.008) and lower H⁺ secretion (28.4 ± 2.4 versus 41.3 ± 4.0 pmol/mm per min; P = 0.016). In Figure 7, HiPro rats that ingested L-NAME had greater distal nephron HCO₃ secretion (−7.2 ± 0.7 versus −3.5 ± 0.4 pmol/mm per min; P < 0.001), but distal nephron net HCO₃ reabsorption (31.7 ± 3.0 versus 37.8 ± 3.2 pmol/mm per min; P = 0.19) and H⁺ secretion (38.8 ± 3.7 versus 41.3 ± 4.0 pmol/mm per min; P = 0.46) were similar to HiPro rats that did not ingest L-NAME. Figure 8 shows that distal nephron net HCO₃ reabsorption (19.9 ± 1.8 versus 22.4 ± 1.9 pmol/mm per min; P = 0.36), HCO₃ secretion (−7.1 ± 0.8 versus −6.0 ± 0.7 pmol/mm per min; P = 0.32), and H⁺ secretion (26.8 ± 2.5 versus 28.4 ± 2.4 pmol/mm per min; P = 0.65) were similar in HiPro rats that simultaneously ingested bosentan+L-NAME compared with those that ingested bosentan alone.

Discussion

Our studies tested the hypothesis that endothelin-induced increased NO production mediates the decreased distal nephron HCO₃ secretion component of augmented distal nephron acidification that is induced by increased dietary protein (HiPro). The data show that HiPro rats had increased urine excretion of metabolic products of NO metabolism that was ameliorated by endothelin receptor antagonism. In addition, the data show that NOS inhibition reduced urine NAE and
increased late distal nephron HCO₃ delivery in HiPro rats. This increased late distal nephron HCO₃ delivery that was induced by NOS inhibition was mediated by increased distal nephron HCO₃ secretion. Furthermore, distal nephron HCO₃ secretion was not different in HiPro rats that underwent simultaneous endothelin receptor antagonism and NOS inhibition compared with HiPro rats that underwent endothelin receptor antagonism alone. The data support that endothelin-stimulated increased NO production mediates the decreased distal nephron HCO₃ secretion component of augmented distal nephron acidification that occurs in response to HiPro.

Increased intake of dietary protein that is composed of acid-producing amino acids such as casein increases metabolic acid production and leads to increased renal acid excretion (18) that is mediated by augmented distal nephron acidification (1,2,19). Augmented distal nephron acidification is manifest by increased net HCO₃ reabsorption (20) that might be mediated by increased H⁺ secretion and/or reduced HCO₃ secretion (10). Increased H⁺ secretion enhances distal nephron acidification through reclaiming filtered HCO₃ and promoting NH₄⁺ secretion (21). In addition, increased distal nephron H⁺ secretion limits terminal nephron HCO₃ delivery that also promotes NH₄⁺ secretion (21) by permitting secreted H⁺ to titrate non-HCO₃ buffers, leading to net acid excretion rather than HCO₃ reclamation (22). Reduced distal nephron HCO₃ secretion also limits terminal nephron HCO₃ delivery with its attendant benefits to renal acidification. Both increased H⁺ secretion and decreased HCO₃ secretion mediate increased distal nephron acidification induced by dietary intake of mineral acids (23), and each component of enhanced distal nephron acidification in this model is endothelin mediated (24). Our previous investigations as to the mechanism(s) for HiPro-induced augmented distal nephron acidification revealed augmented distal nephron H⁺ secretion that is due to endothelin-stimulated increased Na⁺/H⁺ exchanger and increased H⁺-ATPase activity (1,2), the latter as a result of endothelin-stimulated increased aldosterone activity (2). These studies further elucidate the cascade by which HiPro augments distal nephron acidification by showing that the previously demonstrated decrease in distal nephron HCO₃ secretion in HiPro rats (1,2) is mediated through increased NO that in turn is stimulated by endothelin. This increased distal nephron acidification limits the increased HCO₃ delivery to the terminal distal nephron that might otherwise occur in response to increased early distal HCO₃ delivery in situ (as a result of higher distal nephron fluid flows in HiPro rats as discussed in the Materials and Methods section) in HiPro rats compared with control animals (Table 3). Consequently, renal endothelins play an important mediating role in the increased H⁺ secretion/decreased HCO₃ secretion components of augmented distal nephron acidification that is induced by these two models of augmented distal nephron acidification, dietary intake of mineral acid and of acid-producing proteins.

The decreased distal nephron HCO₃ secretion that is induced by HiPro might help ameliorate the obligate HCO₃ secretion that occurs in response to increased fluid flows in this nephron segment (25) and thereby ameliorate the increased terminal
HCO₃ delivery that might otherwise occur with the increased distal nephron fluid flows that are induced by HiPro (1). Decreased distal nephron HCO₃ secretion that is mediated by endothelins also seems to ameliorate the increase in terminal HCO₃ delivery in distal nephrons of remnant kidneys that might otherwise occur as a result of increased distal nephron fluid flows that also characterizes this setting (26). Together, the data suggest that increased NO action that is stimulated by endothelins limits HCO₃ secretion with its attendant decreased acidification that would otherwise occur in these two settings of increased distal nephron fluid flow (remnant kidneys and HiPro).

These studies support that NO enhances distal nephron acidification as supported by some (7) but not all (27) previous investigations. The latter study showed that NO donors inhibit distal nephron H⁺/H₁₅₅₅₅₅₅ ATPase activity and that this effect was prevented by NOS inhibition (27). Whether the differences between the latter and our studies are due to the probable supraphysiologic NO levels induced by NO donors, to the in vitro preparation used (compared with the in vivo preparation of our studies), or to other differences are not known. Our studies are consistent with the former studies, showing that NOS inhibition with L-NAME limits urine and distal nephron acidification in animals that are given an acid challenge. In the former studies, this acid challenge was provided by dietary NH₄Cl, whereas in these studies, the challenge was provided by increased ingestion of acid-producing protein (casein). The former and present studies support an important role of NO in mediating the augmented distal nephron acidification induced by a systemic acid challenge. These studies suggest that NO increases distal nephron acidification in this setting by reducing HCO₃ secretion in this nephron segment. Whether decreased distal nephron HCO₃ secretion that is mediated by NO in this setting is due to modulation of the actions of existing membrane transporters that secrete HCO₃ and/or by affecting the number and membrane location of these transporters was not determined. A chloride-bicarbonate exchanger in the brush border membrane of mammalian duodenal crypt cells mediates HCO₃ secretion by this intestinal segment (28), and NOS inhibition with L-NAME stimulates its activity (29). These data are consistent with NO-mediated inhibition of HCO₃ secretion in this epithelium. Increased NO activity that is induced by augmented endothelin activity might similarly inhibit chloride-bicarbonate exchange–mediated HCO₃ secretion in the mammalian distal nephron in response to increased dietary intake of acid-producing protein such as casein. This NO effect might be mediated directly or indirectly. Figure 9 shows a proposed cascade by which HiPro increases urine NAE that is supported by our series of studies to date.

Although endothelin is an important mediator of increased distal nephron acidification that is induced by HiPro, endothelin A/B receptor antagonism did not restore acidification to control levels in this or our previous studies (1,2). These data suggest that additional mechanisms contribute to augmented distal nephron acidification in HiPro rats. HiPro increases renal angiotensin II activity (30), and angiotensin II increases distal nephron acidification in vivo (31). Consequently, increased angiotensin II activity might contribute to the increment in distal nephron acidification that is not mediated by endothelin.

**Conclusion**

These studies show that HiPro as purified casein augments distal nephron acidification as a result in part of reduced HCO₃ secretion that is mediated by increased NO.

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