Endothelin-Induced Increased Aldosterone Activity Mediates Augmented Distal Nephron Acidification as a Result of Dietary Protein

Apurv Khanna,* Jan Simoni,† and Donald E. Wesson*‡
Departments of *Internal Medicine, †Surgery, and ‡Physiology, Texas Tech University Health Sciences Center, Texas Tech University School of Medicine, Lubbock, Texas

The hypothesis that increased dietary protein augments distal nephron acidification through endothelin-mediated increased aldosterone activity was tested. Munich-Wistar rats were studied after 3 wk of diets with 50% high protein (HiPro) and 20% control (CON) casein-provided protein, the latter comparable to standard diet. HiPro versus CON rats had higher distal nephron H+ secretion by in vivo microperfusion as shown previously. Perfusion with inhibitors of Na+/H+ exchange (EIPA, 10−5 M), H+-ATPase (bafilomycin, 10−7 M), and H+-K+-ATPase (Sch 28080 [10−8 M] and ouabain [10−3 M]) support that higher Na+/H+ exchange and higher H+-ATPase but not higher H+-K+-ATPase activity mediated increased H+ secretion in HiPro rats. Oral bosentan, an endothelin A/B receptor antagonist, decreased distal nephron H+ secretion in HiPro rats as a result of reduced Na+/H+ exchange and H+-ATPase activity as shown previously by the authors’ laboratory. HiPro versus CON rats had higher plasma aldosterone (60.9 ± 5.9 versus 42.2 ± 4.4 pg/ml; P < 0.024) and higher urine aldosterone excretion (21.9 ± 3.9 versus 10.5 ± 2.8 ng/d; P < 0.04) in the absence but not presence of bosentan, consistent with endothelin-mediated increased aldosterone secretion. HiPro rats that did versus did not ingest the endothelin antagonist spironolactone had lower distal nephron H+ secretion (29.2 ± 3.3 versus 42.1 ± 3.8 pmol/mm per min; P < 0.05) as a result of lower H+-ATPase activity without differences in Na+/H+ exchange or H+-K+-ATPase activity. The data support that dietary protein provided as casein increases distal nephron acidification through endothelin-stimulated Na+/H+ exchange and endothelin-stimulated aldosterone secretion that increases H+-ATPase activity.

Dietary acid as ammonium (NH4+) salts augments distal nephron acidification (1,2) through endothelin-dependent mechanisms (3). Dietary intake of acid-producing protein also increases distal nephron acidification (4,5) by mechanisms that are also endothelin dependent (5). In the latter studies, augmented distal nephron acidification was due to an endothelin-mediated increase in both Na+/H+ exchange and H+-ATPase activity (5). Endothelin stimulates Na+/H+ exchange in vitro (6–8), but no studies report that endothelin increases in vitro H+-ATPase activity. Consequently, endothelin might stimulate H+-ATPase activity indirectly given the many endocrine alterations induced by a systemic acid challenge, including stimulation of the renin-angiotensin-aldosterone axis (9,10). Because increased dietary protein increases serum aldosterone (11), aldosterone increases distal nephron H+-ATPase activity (12), and endothelin infusion increases aldosterone secretion in vivo (13,14), the present studies tested the hypothesis that the endothelin-mediated increase in distal nephron H+-ATPase activity induced by acid-producing dietary protein is due to increased aldosterone activity.

Materials and Methods

Animals and Diet Protocol

Male and female Munich-Wistar rats (Harlan Sprague-Dawley, Houston, TX; 200 to 220 g) ate standard rat chow (Frolab RMH 2500 with 23% protein) 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. High protein (HiPro) and control (CON) rats ate 50 and 20% protein, respectively. 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 and complete intake. Some ingested bosentan (Actelion, Allschwil, Switzerland), a nonpeptide endothelin A/B receptor antagonist (15), mixed with study diet (100 mg/kg body wt per d). This oral dose blocks action of pressor doses of intravenous big endothelin-1 (ET-1) for >24 h (15). Others ingested spironolactone (400 mg/kg body wt per d) dissolved in olive oil and mixed with diet. This oral dose is in excess of that required for virtually complete mineralocorticoid receptor blockade in vivo (16). All drank distilled H2O ad libitum.

Urine Net Acid and ET-1 Excretion

We measured daily urine net acid excretion (NAE) (17) and ET-1 (3) in a 24-h sample collected as described (5) from eight each of HiPro and CON rats in metabolic cages. We examined the effect of endothelin and aldosterone receptor blockade with bosentan and spironolactone, respectively, on urine NAE in paired and separate groups of eight each.

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Address correspondence to: Dr. Donald E. Wesson, Texas Tech University Health Sciences Center, Renal Section, 3601 Fourth Street, Lubbock, TX 79430. Phone: 806-743-3107; Fax: 806-743-3177; E-mail: donald.wesson@ttuhsc.edu

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respectively. Separate superficial distal nephrons of HiPro and CON rats by ultrafluorometry (see below) to measure net HCO3 reabsorption (HCO3 passive permeability (19). After the micropunctured (left) kidney was weighed, perfused nephron length was determined by measuring the length of a latex cast injected after micropuncture, recovered after acid digestion of the kidney (19). We measured [HCO3] in stellate vessel plasma to determine peritubular blood-to-lumen HCO3 permeability (19). The perfusion solutions used are shown in Table 1. Solution 1 contained 5 mM HCO3 and 40 mM Cl− to approximate their concentrations at the early distal nephron in situ (21). Solution 2 contained Cl− but no HCO3 to measure Cl−-dependent luminal HCO3 accumulation and to calculate an “apparent” blood-to-lumen H+/HCO3 permeability (19). Solution 3 was HCO3− and Cl−-free and contained 0.5 mM acetazolamide to inhibit transtubule H+/HCO3 transport and was used to determine “passive” blood-to-lumen H+/HCO3 permeability (19). We used this passive permeability determined using solution 3 to calculate passive blood-to-lumen HCO3 secretion when perfusing with the HCO3-containing solution 1 (19). We used the apparent blood-to-lumen H+/HCO3 permeability determined from perfusion with solution 2 to calculate “total” HCO3 secretion when perfusing with HCO3-containing solution 1 (19). We subtracted calculated passive HCO3 secretion from calculated total HCO3 secretion to obtain “net” HCO3 secretion when perfusing with solution 1 (19). The HCO3 secretion reported herein is the net HCO3 secretion that excludes the passive HCO3 secretion calculated as described above. Distal nephron H+ secretion was calculated by subtracting the calculated net HCO3 secretion (a negative value) from the measured net HCO3 reabsorption (HCO3 perfused into the distal nephron minus HCO3 collected) (18). All perfusing solutions contained raffinose to minimize fluid transport and gluconate substituted for Cl− when necessary (19). Each surface distal nephron was perfused with each perfusing solution in the following order: 1, 2, 3. Previous studies showed that the order of perfusing solutions did not affect calculations of the components of distal nephron HCO3 reabsorption (19).

Identification of H+ Transport Mediators of HiPro-Induced Changes in Distal Nephron Acidification

We compared the net decrease in distal nephron H+ secretion in response to specific H+ transport inhibitors to determine contributions of individual distal nephron H+ transporters to differences in overall distal nephron H+ secretion in HiPro versus CON rats. The contribution of Na+/H+ exchange and H+-ATPase was determined by perfusing with solutions that contained EIPA (10−5 M) and bafilomycin (10−7 M), respectively, as done previously in our laboratory (22). Because the rat renal cortex contains the gastric isoform of H+-K+-ATPase (23), we perfused tubules with Sch 28080 (10−5 M) to inhibit this transporter (24) and determine whether its activity was increased in HiPro rats. Although the colonic isoform of H+-K+-ATPase has not been identified in the rat renal cortex (25) and its presence has not been induced by dietary NH4Cl (25), we additionally perfused distal nephrons with solutions that contained both Sch 28080 (10−5 M) and ouabain (10−5 M), the latter inhibiting the colonic H+-K+-ATPase isoform (24). The latter perfusion was done to determine whether HiPro induced and/or increased activity of either the gastric or colonic H+-K+-ATPase isoforms. Greater inhibitor-induced decrease in H+ secretion in HiPro versus CON rats determined increased activity of the H+ transporter inhibited by that compound (22).

Analytical Methods

Immediately after experiment termination, initial and collected perfusate and stellate vessel plasma samples were analyzed for inulin (19) and for TCO2 using flow-through ultrafluorometry (26) as described previously (27). All tubule fluid and plasma TCO2 were measured on the experimental day by comparing fluorescence of a 7- to 8-nl sample aliquot (corrected for a distilled H2O blank run with each sample group) with a standard curve as described previously (27). This technique actually measures TCO2, but we refer to this measured value as HCO3 for simplicity.

Statistical Analyses

Data were expressed as means ± SEM. One to two distal nephron segments were perfused per animal. When two tubules were perfused in an animal, results were averaged to yield a single value for that animal. Group number is number of animals. 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 and Receptor Antagonists on Animal/Kidney/Tubule Growth

The HiPro protocol increases body and kidney weight as well as the length of the accessible distal tubule (5). Neither the endothelin A/B receptor antagonist bosentan nor the mineralocorticoid antagonist spironolactone affected animal/kidney weight, tubule length, food intake, or urine output in either group.

Table 1. Perfusate composition (mM)

<table>
<thead>
<tr>
<th></th>
<th>Solution 1</th>
<th>Solution 2</th>
<th>Solution 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na+</td>
<td>61</td>
<td>61</td>
<td>61</td>
</tr>
<tr>
<td>K+</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Cl−</td>
<td>40</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>HCO3−</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gluconate</td>
<td>20</td>
<td>25</td>
<td>65</td>
</tr>
<tr>
<td>Acetazolamide</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>Raffinose</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
</tbody>
</table>
Effect of HiPro on Arterial Acid-Base Parameters of Conscious Animals

Table 2 shows that HiPro compared with CON arterial pH, \( \text{Pco}_2 \), and calculated [\( \text{HCO}_3^- \)] by blood gases were not different but plasma TCO2 by ultrafluorometry was lower in HiPro. Mean BP (MBP) was not different in HiPro and CON rats (111.5 ± 2.4 versus 108.3 ± 2.1 mmHg; \( P = 0.33; n = 8 \) animals each).

Effect of HiPro on Renal Acidification

HiPro rats have higher urine NAE than CON rats mediated by higher urine \( \text{NH}_4^+ \) and lower \( \text{HCO}_3^- \) excretion with similar excretion of titratable acid (5). Distal nephron net \( \text{HCO}_3^- \) reabsorption was higher in HiPro than CON rats perfused at 6 and 9 nl/min as a result of higher H\(^+\) secretion and to less extent to lower \( \text{HCO}_3^- \) secretion (5).

Transport Mediators of Enhanced Distal Nephron Acidification in HiPro Rats

Figure 1 shows greater net decrease in distal nephron H\(^+\) secretion in HiPro than in CON rats with EIPA (−15.5 ± 1.0 versus −10.5 ± 0.9 pmol/mm per min [\( P < 0.003 \) at 6 nl/min, \( n = 8 \) animals each]) and −15.9 ± 1.1 versus −9.8 ± 0.9 pmol/mm per min [\( P < 0.001 \) at 9 nl/min, \( n = 8 \) animals each]) and bafilomycin (−14.5 ± 1.1 versus −7.7 ± 0.6 pmol/mm per min [\( P < 0.001 \) at 6 nl/min, \( n = 8 \) animals each]) and −12.4 ± 1.0 versus −6.1 ± 0.9 pmol/mm per min [\( P < 0.001 \) at 9 nl/min, \( n = 8 \) animals each]), consistent with enhanced Na\(^+\)/H\(^+\) exchange and increased H\(^+\)-ATPase activity, respectively. Neither Sch 28080-induced (−3.4 ± 0.9 versus −2.8 ± 0.8 pmol/mm per min [\( P = 0.52 \) at 6 nl/min, \( n = 8 \) animals each]) and −4.0 ± 0.8 versus −3.3 ± 0.7 pmol/mm per min [\( P = 0.29 \) for 9 nl/min, \( n = 8 \) animals each]) or Sch 28080+ouabain-induced (−3.1 ± 1.0 versus −1.8 ± 0.9 pmol/mm per min [\( P = 0.07 \) at 6 nl/min, \( n = 4 \) animals each]) and −3.5 ± 0.9 versus −2.7 ± 0.8 pmol/mm per min [\( P = 0.53 \) for 9 nl/min, \( n = 4 \) animals each]) net decrease in H\(^+\) secretion differed in HiPro and CON rats, consistent with no increased H\(^+\)-K\(^+\)-ATPase activity in HiPro rats.

Effect of HiPro on Renal ET-1 and Aldosterone Production

HiPro increases urine ET-1 excretion, ET-1 addition to renal cortical microdialysate, and renal cortical mRNA (5). Figure 2 shows that HiPro compared with CON rats had higher plasma aldosterone (60.9 ± 5.9 versus 42.2 ± 2.4 pg/ml; \( P = 0.033, \) ANOVA; \( n = 8 \) animals each) and higher urine aldosterone excretion (21.9 ± 3.5 versus 10.5 ± 1.8 ng/d; \( P = 0.035, \) ANOVA; \( n = 8 \) animals each) in the absence of bosentan. By contrast, plasma aldosterone (51.4 ± 4.9; \( P = 0.34 \) versus CON; \( n = 8 \) animals) and urine aldosterone excretion (13.0 ± 2.7; \( P = 0.45 \) versus CON; \( n = 8 \) animals) were not different between bosentan-ingesting HiPro and CON rats, respectively.

Effect of Endothelin and Mineralocorticoid Receptor Blockade on Arterial Blood and Urine Parameters

Bosentan did not affect CON arterial plasma acid-base parameters (data not shown). Plasma TCO2, urine net NAE, and MBP were not different in HiPro rats that did compared with did not ingest bosentan (5). By contrast, CON rats that ingested spironolactone had lower plasma TCO2 than concurrently studied CON rats that did not ingest spironolactone (22.9 ± 0.6 versus 25.2 ± 0.6 mM; \( P = 0.017; n = 8 \) animals each) but similar urine NAE (3569 ± 448 versus 4460 ± 639 \( \mu \)M/d; \( P = 0.27; n = 8 \) animals each) and MBP (170.8 ± 2.2 versus 110.1 ± 2.2 mmHg; \( P = 0.74; n = 8 \) animals each). Similarly, HiPro rats that did compared with concurrently studied HiPro rats that did not ingest spironolactone had lower plasma TCO2 (20.2 ± 0.8 versus 23.0 ± 0.5 mM; \( P = 0.02; n = 8 \) animals each) but similar urine NAE (6210 ± 589 versus 7067 ± 937 \( \mu \)M/d; \( P = 0.45; n = 8 \) animals each) and MBP (110.7 ± 2.2 versus 111.5 ± 2.4 mmHg; \( P = 0.81; n = 8 \) animals each).

Effect of Endothelin and Mineralocorticoid Receptor Blockade on HiPro-Induced Augmented Distal Nephron Acidification

Distal nephron acidification was not different between CON rats that did and did not receive bosentan (5). Similarly, distal nephron net \( \text{HCO}_3^- \) reabsorption was not different between CON rats that did and did not receive spironolactone whether perfused at 6 nl/min (10.8 ± 1.7 versus 12.9 ± 1.4 pmol/mm per min, respectively; \( P = 0.36; n = 8 \) animals each) or 9 nl/min (12.8 ± 1.9 versus 14.9 ± 1.4 pmol/mm per min; \( P = 0.39; n = 8 \) animals each). By contrast, HiPro rats that did compared with did not receive bosentan had lower distal tubule net \( \text{HCO}_3^- \) reabsorption that was mediated by lower H\(^+\) secretion and higher \( \text{HCO}_3^- \) secretion (5). Similarly, Figure 3 shows that HiPro rats that did compared with did not receive spironolactone had lower distal nephron net \( \text{HCO}_3^- \) reabsorption (26.7 ± 3.0 versus 39.0 ± 3.1 pmol/mm per min; \( P < 0.013; n = 8 \) animals each). Like the decreased distal nephron net \( \text{HCO}_3^- \) reabsorption as a result of bosentan, the decreased distal nephron net \( \text{HCO}_3^- \) reabsorption as a result of spironolactone was mediated by reduced H\(^+\) secretion (29.2 ± 3.3 versus 42.1 ± 3.8 pmol/mm per min; \( P < 0.023; n = 8 \) animals each). Unlike decreased distal nephron net \( \text{HCO}_3^- \) reabsorption induced by bosentan that was also mediated by increased distal nephron \( \text{HCO}_3^- \) secretion (5), these parameters (data not shown).

### Table 2. Plasma acid-base data in conscious animals after 3 weeks of dietary protein

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>( \text{Pco}_2 ) (mmHg)</th>
<th>Calculated [( \text{HCO}_3^- )] (mM)</th>
<th>Measured [( \text{TCO}_2 )] (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>7.41 ± 0.02</td>
<td>39.2 ± 1.0</td>
<td>24.1 ± 0.9</td>
<td>25.2 ± 0.6</td>
</tr>
<tr>
<td>HiPro</td>
<td>7.38 ± 0.02</td>
<td>38.5 ± 1.0</td>
<td>22.1 ± 0.8</td>
<td>23.3 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values are means ± SEM. CON, control; HiPro, 3 wk of 50% dietary protein; TCO2, total CO2.

<sup>b</sup>\( P < 0.05 \) versus 20% protein.
this component of distal nephron net HCO₃ reabsorption was not different in HiPro rats that did compared with did not ingest spironolactone (−5.4 ± 0.6 versus −3.9 ± 0.7 pmol/mm per min; P = 0.13; n = 8 animals each).

Effect of Endothelin and Mineralocorticoid Receptor Blockade on Enhanced H⁺ Transporter Activity Induced by HiPro

Net decrease in distal nephron H⁺ secretion was not different in bosentan-ingesting compared with noningesting HiPro rats that were perfused at 9 nl/min with EIPA and bafilomycin, consistent with no additional effect of these inhibitors on Na⁺/H⁺ exchange and H⁺-ATPase activity, respectively, in HiPro rats with endothelin A/B receptor blockade (5). There was also no difference in the net decrease in H⁺ secretion in bosentan-ingesting compared with noningesting HiPro rats that were perfused with Sch 28080 (5), consistent with no effect of endothelin A/B receptor blockade on H⁺-K⁺-ATPase activity in HiPro rats. By contrast, Figure 4 shows that spironolactone-ingesting compared with noningesting HiPro rats had less bafilomycin-induced decrease in distal nephron H⁺ secretion when perfused at 9 nl/min (−6.5 ± 1.2 versus −14.5 ± 1.1 pmol/mm per min; P < 0.001; n = 8 animals each), consistent with spironolactone-induced inhibition of the previously demonstrated augmented H⁺-ATPase activity of HiPro rats. By contrast, EIPA-induced (−13.2 ± 1.4 versus −15.9 ± 1.1 pmol/mm per min; P = 0.15 at 9 nl/min; n = 8 animals each), Sch 28080-induced (−6.1 ± 0.9 versus −4.0 ± 0.8 pmol/mm per min; P = 0.10 at 9 nl/min; n = 8 animals each), and Sch 28080 + ouabain-induced (−8.9 ± 1.6 versus −6.6 ± 0.9 pmol/mm per min; P = 0.26 at 9 nl/min; n = 4 animals each) decrease in distal nephron H⁺ secretion was not different in spironolactone-ingesting compared with noningesting HiPro rats, consistent with no spironolactone effect on Na⁺/H⁺ exchange or H⁺-K⁺-ATPase activity, respectively, in HiPro rats.
Discussion

These studies tested the hypothesis that the endothelin-dependent increase in distal nephron \( \text{H}^+ \)-ATPase activity in HiPro rats (5) is mediated through endothelin-stimulated aldosterone secretion that enhances \( \text{H}^- \)-ATPase activity. HiPro as casein was associated with increases of plasma aldosterone and urine aldosterone excretion in the absence but not presence of the endothelin A/B receptor antagonist bosentan. Furthermore, reduction in distal nephron \( \text{H}^- \) secretion induced by the \( \text{H}^- \)-ATPase inhibitor bafilomycin was less in the presence than absence of the aldosterone receptor antagonist spironolactone. Together, the present studies support that HiPro as casein induces endothelin-stimulated secretion of aldosterone that increases distal nephron \( \text{H}^- \)-ATPase activity. Because increased dietary protein induces endothelin-mediated increased distal nephron \( \text{H}^- / \text{H}^- \) exchange (5), augmented \( \text{H}^- \) secretion induced by HiPro is mediated by the combined effects of endothelin and aldosterone.

Increased intake of dietary protein that is composed of acid-producing amino acids increases metabolic acid production and renal acid excretion (28) that is mediated by augmented distal nephron acidification (4,5). Augmented distal nephron acidification is manifest by increased net \( \text{HCO}_3^- \) reabsorption (29), consistent with increased \( \text{H}^- \) secretion that reclaims filtered \( \text{HCO}_3^- \) and promotes \( \text{NH}_4^- \) secretion (30). The resulting decrease in \( \text{HCO}_3^- \) delivery to the terminal nephron also promotes \( \text{NH}_4^- / \text{H}^- \) exchange and increased \( \text{H}^- \)-ATPase activity (5). The previous (5) and present studies support that this enhanced \( \text{Na}^+ / \text{H}^- \) exchange and \( \text{H}^- \)-ATPase activity is mediated by endothelin and aldosterone, respectively. Augmented distal nephron acidification induced by dietary acid is also due to decreased distal nephron \( \text{HCO}_3^- \) secretion (2), again through an endothelin-dependent mechanism (3). Endothelin infusion decreases distal nephron \( \text{HCO}_3^- \) secretion (32), but whether this effect is direct or is mediated through another agent was not determined. Consequently, endothelins play an important role in mediating both the increased \( \text{H}^- \) secretion and decreased \( \text{HCO}_3^- \) secretion induced by dietary acid that mediate the increased distal nephron acidification in this setting.

Spironolactone was used as the aldosterone antagonist in the present studies to demonstrate the aldosterone-mediated increase in distal nephron \( \text{H}^- \)-ATPase activity. Other investigators re-
ported studies supporting that spironolactone increases endo-
gous acid production (33) that might have contributed to the
decrease in plasma TCO2 observed in both HiPro and CON ani-
mals that ingested spironolactone. The microperfusion studies
reported in the present studies nevertheless support that aldoste-
ronemediated increased H⁺-ATPase activity contributed to the
augmented H⁺ secretion of HiPro animals.

The mechanisms by which dietary acid as NH₄Cl salts (3) or
as acid-producing dietary protein (4,5) increases renal endothe-
lin production and cell type that is the endothelin source are not
clear. Renal microvascular endothelial cells that are exposed to
an acid extracellular environment in vivo increase ET-1 release
into the culture medium (34), possibly contributing to increased
renal interstitial fluid ET-1 levels in response to dietary acid (3).
Nevertheless, renal epithelial cells also make and secrete ET-1
(35) and might also be a source of the augmented renal endo-
thelin. The present studies support that the increased plasma
and urine aldosterone associated with HiPro was mediated by
endothelin. Similarly, chronic dietary NH₄Cl increases plasma
aldosterone (36), and endothelin might also mediate the in-
creased plasma aldosterone that occurs in this setting. Endo-
thelin infusion increases aldosterone secretion in rats in vivo
(13,14), and endothelin is an important paracrine regulator of
zone glomerulosa function (37).

In summary, HiPro as purified casein augments distal
nephron H⁺ secretion through aldosterone-stimulated in-
creased H⁺-ATPase activity. The increased aldosterone in this
setting is mediated through endothelin, which itself increases
distal nephron Na⁺/H⁺ exchange. The data support that aldo-
sterone and endothelins are important mediators of the in-
creased distal nephron acidification associated with increased
intake of dietary protein, the common dietary challenge to
systemic acid-base homeostasis faced by humans.

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