Angiotensin II Activates H⁺-ATPase in Type A Intercalated Cells

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ABSTRACT

We reported previously that angiotensin II (AngII) increases net Cl⁻/H⁺ absorption in mouse cortical collecting duct (CCD) by transcellular transport across type B intercalated cells (IC) via an H⁺-ATPase– and pendrin-dependent mechanism. Because intracellular trafficking regulates both pendrin and H⁺-ATPase, we hypothesized that AngII induces the subcellular redistribution of one or both of these exchangers. To answer this question, CCD from furosemide-treated mice were perfused in vitro, and the subcellular distributions of pendrin and the H⁺-ATPase were quantified using immunogold cytochemistry and morphometric analysis. Addition of AngII in vitro did not change the distribution of pendrin or H⁺-ATPase within type B IC but within type A IC increased the ratio of apical plasma membrane to cytoplasmic H⁺-ATPase three-fold. Moreover, CCDs secreted bicarbonate under basal conditions but absorbed bicarbonate in response to AngII. In summary, angiotensin II stimulates H⁺ secretion into the lumen, which drives Cl⁻ absorption mediated by apical Cl⁻/HCO₃⁻ exchange as well as generates more favorable electrochemical gradient for ENaC-mediated Na⁺ absorption.


The cortical collecting duct (CCD) consists of principal and intercalated cells (IC; Figure 1) and contributes to the regulation of acid-base and fluid and electrolyte balance.1 Within the CCD, most net Na⁺ absorption occurs across principal cells through the epithelial Na⁺ channel (ENaC).2 In contrast, IC mediate secretion of OH⁻/H⁺ equivalents and net Cl⁻ absorption.3,4 Within mouse CCD, IC are subclassified on the basis of the location of the H⁺-ATPase and the presence or absence of the Cl⁻/HCO₃⁻ exchanger anion exchanger 1 (AE1). Type A IC (A-IC) mediate H⁺ secretion and express the H⁺-ATPase on the apical plasma membrane and AE1 on the basolateral plasma membrane, whereas type B IC (B-IC) express the H⁺-ATPase on the basolateral plasma membrane.5 The apical plasma membrane of type B cells expresses pendrin, a Na⁺-independent Cl⁻/HCO₃⁻ exchanger encoded by Slc26a4, which mediates Cl⁻ absorption and secretion of OH⁻ equivalents, such as HCO₃⁻.6,7 The apical plasma membrane of non-A, non-B-IC expresses both pendrin and the H⁺-ATPase; however, this cell subtype is very rare in mouse CCD.9

Although the mechanism of Na⁺ absorption has been elucidated in detail, the molecular mechanism(s) responsible for Cl⁻ transport are less well understood. Cl⁻ absorption occurs primarily through transcellular transport across B-IC with a small contribution of a paracellular, conductive flux.3,4,10–12 Cl⁻ enters B cells, at least in part, through the apical Cl⁻/HCO₃⁻ exchanger, pendrin, and likely exits the cells across the basolateral plasma membrane through a Cl⁻ conductance.3,10 Within mouse CCD, we observed that angiotensin II (AngII) increases Cl⁻ absorption by transcellular transport across B-IC through a pendrin-
an H⁺-ATPase–dependent mechanism; therefore, we hypothesized that AngII either increases apical plasma membrane pendrin expression or increases the driving force for apical Cl⁻/HCO₃⁻ exchange, such as by activating the H⁺-ATPase. Because intracellular trafficking is a major mechanism by which both pendrin and the H⁺-ATPase are regulated, we asked whether AngII induces subcellular redistribution of pendrin and/or the H⁺-ATPase. To answer this question, we performed immunogold cytochemistry and ion transport studies in mouse CCD perfused in vitro.

**RESULTS**

**Within Type B Cells, AngII Does not Alter the Subcellular Distribution of Pendrin or the H⁺-ATPase**

We asked whether AngII application in vitro increases Cl⁻ absorption by increasing apical plasma membrane pendrin expression. To answer this question, CCD were perfused in vitro from mice given a NaCl-replete diet and furosemide for 5 d to upregulate pendrin, H⁺-ATPase, and ENaC protein expression. Pendrin expression was quantified in these tubules with immunogold cytochemistry. Numerous gold particles labeled the cytoplasmic vesicles of the B-IC, whereas only faint background staining was present in the cytoplasm of the adjacent principal cells (Figure 2). As shown (Figure 3, Table 1), a 45-min exposure to AngII did not increase apical plasma membrane pendrin expression in B cells. However, pendrin–mediated Cl⁻ absorption might increase through activation of the basolateral plasma membrane H⁺-ATPase in B cells. Increased net H⁺ efflux, such as through activation of the H⁺-ATPase, should raise intracellular HCO₃⁻ concentration, thereby augmenting the driving force for apical Cl⁻/HCO₃⁻ exchange. Thus, basolateral plasma membrane H⁺-
Table 1. Effect of AngII on cell morphometry and pendrin and H^+ -ATPase subcellular distribution in B-IC

<table>
<thead>
<tr>
<th>B-IC</th>
<th>Pendrin</th>
<th>H^+ -ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n = 3)</td>
<td>AngII (n = 4)</td>
</tr>
<tr>
<td>Apical plasma membrane boundary length, μm</td>
<td>14.6 ± 2.6</td>
<td>13.5 ± 0.3</td>
</tr>
<tr>
<td>Apical plasma membrane immunolabel, gold particles/cell profile</td>
<td>24.9 ± 6.2</td>
<td>20.9 ± 5.0</td>
</tr>
<tr>
<td>Apical plasma membrane pendrin label density, gold particles/μm</td>
<td>1.66 ± 0.13</td>
<td>1.53 ± 0.33</td>
</tr>
<tr>
<td>Basolateral plasma membrane immunolabel, gold particles/cell profile</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Immunolabel over the cytoplasm and cytoplasmic vesicles, gold particles/cytoplasm</td>
<td>249 ± 63</td>
<td>273 ± 53</td>
</tr>
<tr>
<td>Pendrin cytoplasmic label density, gold particles/μm² of cytoplasmic area</td>
<td>4.4 ± 0.7</td>
<td>4.6 ± 0.9</td>
</tr>
<tr>
<td>Pendrin subcellular immunolabel redistribution ratio, apical plasma membrane gold/cytoplasmic gold</td>
<td>0.106 ± 0.021</td>
<td>0.078 ± 0.011</td>
</tr>
<tr>
<td>H^+ -ATPase subcellular immunolabel redistribution ratio, basolateral plasma membrane gold/cytoplasmic gold</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

^H^+-ATPase or pendrin protein abundance was determined by quantifying the number of immunogold particles directed at H^+ -ATPase and pendrin antigen–antibody interactions, respectively. n = number of tubules studied. N/A, not applicable.

**AngII Stimulates HCO₃⁻ Absorption in CCD**

We asked whether AngII increases apical plasma membrane H^+ -ATPase expression within A-IC. As shown, with application of AngII to the bath, the proportion of H^+ -ATPase expressed in the apical plasma membrane relative to the cytoplasm increased more than three-fold (Table 2, Figure 5). Only a few gold particles were present in the cytoplasm of the adjacent principal cells (Figure 6). We concluded that AngII increases apical plasma membrane H^+ -ATPase expression in A-IC through subcellular redistribution.

**DISCUSSION**

We reported previously that AngII stimulates Cl⁻ absorption in the CCD of wild-type but not of Slc26a4 null mice.12 Thus, AngII-induced Cl⁻ absorption occurs through a pendrin-dependent mechanism. Whereas in vivo studies showed that

![Figure 4](image_url)
chronic regulation of pendrin occurs mainly through subcellular redistribution between apical plasma membrane and cytoplasmic vesicles,

this study shows that AngII does not modulate Cl− absorption through pendrin trafficking when applied in vitro at a concentration (10−8 M) observed in the cortical interstitium in vivo. Therefore, AngII-stimulated Cl− absorption must be achieved by other mechanism(s), such as through posttranslational modification of pendrin or through changes in the driving force for apical anion exchange. In this study, we observed that AngII increases apical plasma membrane H+-ATPase expression in A-IC in tandem with increased HCO3− absorption (or increased net H+ secretion). Because activation of the H+-ATPase reduces luminal HCO3− concentration, the driving force for apical Cl−/HCO3− exchange increases, thus increasing net Cl− absorption. H+-ATPase activation is critical in AngII-induced Cl− absorption, because angiotensin does not alter Cl− absorption when the H+-ATPase is inhibited.§

We cannot exclude the possibility, however, that AngII also activates B cell transporters, such as pendrin and the basolateral plasma membrane H+-ATPase, through mechanisms other than trafficking, such as posttranslational modification. Moreover, AngII might increase the driving force for apical anion exchange by a direct effect on basolateral Cl− exit from B cells, such as through changes in KCl co-transporter 1 or changes in a basolateral Cl− channel; however, either primary or secondary activation of apical anion exchange in B cells by AngII should increase luminal bicarbonate concentration, which was not observed in this study. Instead, we observed reduced luminal HCO3− concentration with AngII application.

AngII activates the H+-ATPase in the proximal tubule. In more distal regions of the nephron, however, regulation of the H+-ATPase by AngII remains particularly controversial. In the late distal tubule and in distal nephron cells in culture (MDCK), AngII activates the H+-ATPase. In contrast, Tojo et al. demonstrated a dosage-dependent inhibition of the H+-ATPase by AngII in permeabilized CCD segments; however, Tojo et al. studied the H+-ATPase activity in pooled membrane fragments. Therefore, possible effects of AngII on the subcellular distribution of H+-ATPase could not be discerned.

The primary physiologic role of AngII is to maintain arterial blood pressure through vasconstrictor and by increasing renal Na+ absorption, such as through ENaC activation in the CCD. However, ENaC-mediated Na+ absorption generates a current of positive charge from the lumen to the bath, we expected transepithelial voltage (Vt) to become more lumen negative with AngII. Instead, Vt did not change after application of the hormone. Thus, movement of another ion must shunt the potential generated by ENaC-mediated Na+ absorption. Electrogenic Cl− absorption might shunt the Vt generated by ENaC; however, AngII-stimulated Cl− absorption is largely mediated by pendrin, which is likely an electroneutral exchanger. Although electrogenic K+ secretion attenuates the voltage generated by this Na+ current, Na+ absorbed greatly exceeds K+ secreted. Thus, another ion current must shunt the electrical potential generated by ENaC-mediated Na+ absorption. Because the vacuolar H+-ATPase is an electrogenic transporter, juxtaglomerular H+ secretion is a likely candidate. Simultaneous activation of apical plasma membrane H+-ATPase and ENaC by AngII increases two currents in opposing directions. AngII application thus increases absorption of Na+ and Cl− and increases secretion of H+ equivalents without an

### Table 2. Effect of AngII on cell morphometry and H+-ATPase subcellular distribution in A-ICa

<table>
<thead>
<tr>
<th>Apical plasma membrane boundary length, μm</th>
<th>A-IC</th>
<th>Control (n = 4)</th>
<th>AngII (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apical plasma membrane H+-ATPase label, gold particles/cell</td>
<td>19.3 ± 4.4</td>
<td>32.2 ± 4.2b</td>
<td></td>
</tr>
<tr>
<td>Apical plasma membrane H+-ATPase label density, gold particles/μm</td>
<td>13.9 ± 6.2</td>
<td>35.8 ± 8.3b</td>
<td></td>
</tr>
<tr>
<td>H+-ATPase label over the cytoplasm and cytoplasmic vesicles, gold particles/cytoplasm</td>
<td>0.60 ± 0.20</td>
<td>1.11 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>H+-ATPase cytoplasmic label density, gold particles/μm² of cytoplasmic area</td>
<td>167 ± 34</td>
<td>177 ± 15</td>
<td></td>
</tr>
<tr>
<td>Subcellular H+-ATPase label redistribution ratio, apical plasma membrane gold/cytoplasmic gold</td>
<td>6.3 ± 1.0</td>
<td>7.4 ± 0.6</td>
<td></td>
</tr>
</tbody>
</table>

H+-ATPase label was determined by the number of immunogold particles directed at H+-ATPase antigen-antibody interactions. n = number of tubules studied.

aP = 0.05 to 0.10 versus control.

bP < 0.05.

cP < 0.05.

§This study demonstrates that AngII activates the apical but not the basolateral plasma membrane H+-ATPase; however, we observed previously that application of the H+-ATPase inhibitor bafilomycin to the bath solution abolished AngII-stimulated Cl− absorption. Thus, stimulation of pendrin-dependent Cl− absorption by AngII could be dependent on the basolateral plasma membrane H+-ATPase. However, puterribular application might inhibit both the apical and the basolateral plasma membrane H+-ATPase because bafilomycin is membrane permeable. Thus, the effect of peritubular bafilomycin (10 nM) on AngII-stimulated jCCO2 was explored. If bafilomycin inhibits only H+ efflux across the basolateral plasma membrane of B cells, then it should reduce intracellular HCO3− concentration, thereby attenuating apical anion exchange. The result would be reduced luminal HCO3− concentration and a more positive jCCO2. In contrast, inhibition of H+ secretion by A cells would increase luminal HCO3− concentration, thereby making jCCO2 less positive. We observed that in CCD perfused in vitro from furosemide-treated mice, jCCO2 was −0.1 ± 1.3 pmol/mm per min (n = 6) in the absence and −2.6 ± 1.3 pmol/mm per min (n = 5; P = 0.17) in the presence of bafilomycin in the bath solution. Thus, application of bafilomycin to the bath solution does not reduce HCO3− secretion. One explanation for these data is that peritubular bafilomycin inhibits both the apical and the basolateral plasma membrane H+-ATPase. In contrast, inhibition of H+ secretion by A cells would increase luminal HCO3− concentration, thereby making jCCO2 less positive. We observed that in CCD perfused in vitro from furosemide-treated mice, jCCO2 was −0.1 ± 1.3 pmol/mm per min (n = 6) in the absence and −2.6 ± 1.3 pmol/mm per min (n = 5; P = 0.17) in the presence of bafilomycin in the bath solution. Thus, application of bafilomycin to the bath solution does not reduce HCO3− secretion. One explanation for these data is that peritubular bafilomycin inhibits both the apical and the basolateral plasma membrane H+-ATPase.
appreciable change in $V_T$. These and previously published data are consistent with the hypothesis that the $H^+\text{-ATPase}$ shunts the $V_T$ generated by ENaC-mediated Na$^+$ absorption.

This and previous studies show a novel role of the apical H$^+$-ATPase in AngII-induced NaCl absorption in the CCD. We propose that the H$^+$-ATPase modulates NaCl absorption in this segment through three mechanisms. First, protons secreted by the H$^+$-ATPase titrate luminal HCO$^3$ to produce H$_2$O and CO$_2$. Thus, luminal HCO$_3^\text{-}$ concentration is reduced, which provides a more favorable driving force for apical Cl$^-$/HCO$^3$ exchange-mediated Cl$^-$ absorption. Second, the CO$_2$ thus generated diffuses from the lumen into B cells, which further stimulates apical anion exchange. Third, because the H$^+$-ATPase is an electrogenic transporter, H$^+$-ATPase–mediated H$^+$ secretion should generate a more favorable electromotive force for ENaC-mediated Na$^+$ absorption. The blunted increment in net Cl$^-$ absorption observed in CCD from Slc26a4 null mice in response to AngII likely contributes to the reduced apparent vascular volume and lower BP observed in these mutant mice with furosemide administration or after dietary restriction of NaCl or Cl$^-$ alone.

Using mouse CCD perfused in vitro, we demonstrate that the regulation of pendrin and H$^+$-ATPase expression and function can be studied in native tissue in vitro under identical conditions. Moreover, this approach enables study of transporter trafficking without the changes in protein function that can occur with fluorescent tags.

We conclude that acute application of AngII in vitro increases apical plasma membrane H$^+$-ATPase expression and function specifically in A-IC. Activation of the apical plasma membrane H$^+$-ATPase reduces luminal HCO$^3$ concentration, thus providing an active step for net Cl$^-$ absorption. Moreover, H$^+$ secretion generates a more favorable electromotive force for ENaC-mediated Na$^+$ absorption. Thus, in

We observed previously that AngII does not change $V_T$ in the CCD. To test the hypothesis that the H$^+$-ATPase activation shunts the $V_T$ generated by AngII-induced ENaC activation, we compared $V_T$ measured in the presence and absence of bafilomycin reported previously. In the presence of AngII alone, $V_T$ was $-2.2 \pm 0.7\, \text{mV} (n = 16)$. With AngII plus bafilomycin in the bath, $V_T$ was $-8.0 \pm 1.7\, \text{mV} (n = 6; P < 0.05)$. One explanation of these data is that in the absence of the H$^+$-ATPase–mediated proton secretion, the ENaC-mediated $V_T$ is unmasked.

Figure 5. A cell H$^+$-ATPase immunolabel in the presence and absence of AngII. Representative transmission electron micrographs showing H$^+$-ATPase immunogold labeling in the apical plasma membrane (arrows) and apical cytoplasmic vesicles (arrowheads) in A-IC under control conditions (A) and after application of 10$^{-8}$ M AngII to the bath (B). A-IC exhibited redistribution of the H$^+$-ATPase. H$^+$-ATPase abundance increased in the apical plasma membrane relative to the cytoplasmic vesicles, which was confirmed by quantitative immunogold analyses (see Table 2). As illustrated, an increase in the length and number of apical plasma membrane microprojections was observed in many type A cells in the AngII-treated tubules, although the difference in apical plasma membrane boundary length was not statistically significant (see Table 2).

Figure 6. H$^+$-ATPase immunolabel in A cells and in PC. Transmission electron micrographs of a control isolated perfused CCD labeled for the H$^+$-ATPase by immunogold cytochemistry are shown. (A) Low-magnification image illustrating an A-IC on the right and PC on the left. Bar = 1 $\mu$; *tubule lumen. (B) Higher magnification image of the apical region of cells shown in A. Numerous gold particles label the apical cytoplasmic vesicles and a few particles label the apical plasma membrane of the A-IC. Only rare gold particles are present in the cytoplasm of the PC.
addition to its widely known role in acid-base homeostasis, the H^+·ATPase plays a novel role in NaCl homeostasis. However, the extent to which H^+·ATPase activation contributes to ENaC-mediated Na^+ absorption remains to be determined. Moreover, whether the increased apical plasma membrane H^+·ATPase expression occurs in response to another signal, such as a reduction in intracellular pH (pHi) that results from increased H^+ uptake or increased HCO_3^- efflux across the basolateral plasma membrane, remains to be determined.

**CONCISE METHODS**

**Animals**

All experiments were performed on male and female wild-type mice (strain 129S6/SvEv; Taconic Farms, Germantown, NY). Mice were fed a balanced diet (53881300; Zeigler Brothers, Gardners, PA) prepared as a gel (0.6% agar, 74.6% water, and 24.8% mouse chow) supplemented with NaCl (approximately 1.13 mEq/d NaCl) plus furosemide (100 mg/kg per d) for 5 d to upregulate pendrin, H^+·ATPase, and ENaC expression in CCD.12,17–19 All animal protocols were approved by the Emory University Institutional Animal Care and Use Committee.

**In Vitro Perfusion of Isolated CCD**

CCD were dissected from medullary rays and perfused at flow rates of 2 to 3 nl/min per mm in the presence of symmetric, physiologic solutions containing (in mmol/L) 125 NaCl, 2.5 K_2HPO_4, 24 NaHCO_3/5% CO_2, 2 CaCl_2, 1.2 MgSO_4, and 5.5 glucose bubbled with 95% air/5% CO_2.6,35 Tubules were equilibrated at 37°C for 30 min before the collections were started. Tubules were perfused in the presence and absence of AngII (10^-8 M) applied to the bath and/or bafilomycin A1 applied to the bath (10^-6 M). Stock solutions of AngII (10^-5 M) and bafilomycin (10^-5 M) were prepared in deionized water and in absolute ethanol, respectively. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

**Measurement of JtCO_2**

Total CO_2 (HCO_3^- + H_2CO_3 + CO_2) concentration was measured in perfusate and collected samples using a continuous-flow fluorimeter using the method of Zhou et al.37 Transepithelial total CO_2 flux, JtCO_2, was calculated according to the equation JtCO_2 = (C_0 - C_L)Q/L, where C_0 and C_L are the ion concentrations measured in the perfusate and collected fluid, Q is flow rate in nl/min and L is tubule length. Net fluid transport was taken to be zero because net fluid flux has not been observed in CCD when perfused in vitro in the presence of symmetric solutions and in the absence of vasopressin.38–40 JtCO_2 was expressed in picomoles per millimeter per minute. In each tubule studied, measurements were made under only a single experimental condition.

**Fixation of Tubules Perfused In Vitro**

Separate tubules were studied in flux and immunogold cytochemistry experiments. CCD were perfused in vitro as indicated previously with or without AngII in the bath for 45 min at 37°C, which represents the midpoint at which collections were made, and then fixed. To do so, both perfusate and bath were quickly exchanged for fixative, which contained the perfusate solution and 1% glutaraldehyde. Tubules were then disconnected from the pipettes and transferred into a glass well and incubated in fixative for 1 h at 4°C. Tubules were then trapped in 2% agar and immersed in PBS (pH 7.4), stored at 4°C, and shipped overnight on ice to the University of Florida College of Medicine Electron Microscopy Core Facility.

**Tubule Processing and Immunogold Localization**

The preserved tubules were located under a dissecting microscope, dissected in a cube of agar, dehydrated in a graded series of alcohol, and processed in Lowicryl K4M. The tubules were oriented to the extent possible parallel to the block face to maximize the number of cell profiles included in each ultrathin section. Samples were polymerized under ultraviolet light as described previously.41 Ultrathin sections were cut and labeled for pendrin and the H^+·ATPase as well as every A cell positive for apical plasma membrane pendrin and basolateral plasma membrane H^+·ATPase as well as every A cell pos-
itive for the apical H\(^+\)-ATPase) was photographed and analyzed. IC were photographed at a primary magnification of \( \times 5000 \) and examined at a final magnification of approximately \( \times 18,200 \). The exact magnification was calculated by using a calibration grid with 1134 lines/mm. Apical and basolateral plasma membrane boundary length and cytoplasmic area were determined by point and intersection counting using the Merz curvilinear test grid and standard stereologic formulas.\(^{42} \)

**Antibodies**

The primary polyclonal antibodies recognizing pendrin\(^{6,41} \) and B\(_1\) subunit of vacuolar type H\(^+\)-ATPase,\(^{33} \) as well as the secondary goat anti-rabbit IgG antibody conjugated to 0.8-nm colloidal gold particles (Aurion UltraSmall gold conjugate; Electron Microscopy Sciences, Ft. Washington, PA),\(^{41} \) have been reported previously.

**Statistical Analyses**

All data are presented as means \( \pm \) SEM. Data from two collections from each tubule were averaged to obtain a single value. Each "\( n \)" used in the statistical analysis represents data from an individual tubule. Only one tubule was obtained from a single mouse. For testing for statistical significance between two groups, an unpaired \( t \) test was used. The criterion for statistical significance was \( P < 0.05 \).

**ACKNOWLEDGMENTS**

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**DISCLOSURES**

None.

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