Kidney

A Novel Amiloride-Sensitive H⁺ Transport Pathway Mediates Enhanced Superoxide Production in Thick Ascending Limb of Salt-Sensitive Rats, Not Na⁺/H⁺ Exchange

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Abstract—It has been reported previously that H⁺ efflux via the Na⁺/H⁺ exchange stimulates NAD(P)H oxidase–dependent superoxide (O₂⁻) production in medullary thick ascending limb. We have demonstrated recently that N-methyl-amiloride–sensitive O₂⁻ production is enhanced in the thick ascending limb of Dahl salt-sensitive (SS) rats, suggesting that H⁺ efflux through Na⁺/H⁺ exchangers may promote renal oxidative stress and the development of hypertension in these animals. In the current study we demonstrate, using selective and potent inhibitors, that inhibition of Na⁺/H⁺ exchange does not mediate the ability of N-methyl-amiloride to inhibit thick ascending limb O₂⁻ production. To determine the mechanism of action of N-methyl-amiloride, we examined H⁺ efflux and O₂⁻ production in SS and SS.13BN thick ascending limbs of prehypertensive, 0.4% NaCl–fed rats. Tissue strips containing the medullary thick ascending limb were isolated from male SS and salt-resistant consomic SS.13BN rats, loaded with either dihydroethedium or 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester, and imaged in a heated tissue bath. In Na⁺-replete media, activation of Na⁺/H⁺ exchange using an NH₄Cl prepulse did not stimulate thick ascending limb O₂⁻ production. In Na⁺-free media containing BaCl₂ in which Na⁺/H⁺ activity was inhibited, an NH₄Cl prepulse stimulated O₂⁻ production in medullary thick ascending limb renal tubular segments. This response was enhanced in medullary thick ascending limb of SS rats (slope Δethidium/Δdihydroethedium = 0.029±0.004) compared with SS.13BN rats (slope = 0.010±0.004; P<0.04) and could be inhibited by N-methyl-amiloride (slope=0.005±0.002 and 0.006±0.002 for SS and SS.13BN, respectively). We concluded that only H⁺ efflux through a specific, as-yet-unidentified, amiloride-sensitive H⁺ channel promotes O₂⁻ production in the medullary thick ascending limb and that this channel is upregulated in SS rats. (Hypertension. 2009;54:248-254.)

Key Words: amiloride ■ blood pressure ■ free radicals ■ H⁺ transport ■ kidney ■ NAD(P)H oxidase ■ pH

Superoxide (O₂⁻) production is enhanced in the outer medulla of Dahl salt-sensitive (SS) rats and has been demonstrated to contribute to the development of hypertension in these animals. In a recent study we demonstrated that O₂⁻ production in response to cellular shrinkage was enhanced in medullary thick ascending limb renal tubular segments (mTALs) of SS rats and that N-methyl-amiloride reduced O₂⁻ production in SS mTALs to levels observed in salt-resistant control SS.13BN rats. These findings were consistent with previous findings indicating that amiloride-sensitive O₂⁻ production in mTALs could be driven by H⁺ efflux. Together, these data led us to hypothesize that much of the O₂⁻ production in mTALs is linked to the activity of Na⁺/H⁺ exchange (NHE). To test this hypothesis further, in the current study we used unique derivatives of amiloride capable of selective inhibition of NHE-1 and NHE-3, the predominant isoforms of NHE found in mTALs. Surprisingly, incrementing bath NaCl from 154 to 254 to 500 mmol/L, as we had done in our previous study, in the presence of potent and specific inhibitors of NHE-1 and NHE-3 did not reduce O₂⁻ production in mTALs of SS rats to control levels, indicating that the NHE activity was not mediating extracellular NaCl-induced O₂⁻ production.

Given these data along with the evidence that H⁺ efflux stimulates mTAL NAD(P)H oxidase, we hypothesized that N-methyl-amiloride was inhibiting O₂⁻ production in SS mTALs by blocking a H⁺ transport pathway other than NHE. To test this hypothesis, in the current study we acidified freshly isolated mTALs from SS and SS.13BN rats using the NH₄Cl prepulse technique. O₂⁻ responses and the rate of pH recovery in mTAL were determined as follows: (1) in NaHCO₃-free, Na⁺-replete media in which the majority of H⁺ efflux occurs via NHE; (2) in Na⁺-free media in which NHE was inhibited and H⁺ efflux must occur predominantly...
through secondary H\(^+\) transport pathways other than NHE; and (3) in Na\(^+-\)-free media in the presence of Ba\(^{2+}\) in which many of these secondary H\(^+\) efflux pathways that are not sensitive to amiloride were inhibited. We hypothesized first that only specific activation of a subgroup of H\(^+\) transport pathways other than NHE would result in O\(_2\)^{−} production in response to H\(^+\) efflux in mTAL and that this pathway would be sensitive to inhibition by N-methyl-amiloride. Second, we hypothesized that this novel pathway of amiloride-sensitive O\(_2\)^{−} production was linked to the activity of NAD(P)H oxidase and would be enhanced in the mTAL of SS rats compared with salt-resistant SS.13\(^{BN}\) rats, thus potentially accounting for the oxidative stress and salt sensitivity observed in SS rats.

Methods

Experimental Animals

Studies used 7- to 10-week-old male SS and SS.13\(^{BN}\) rats (The Medical College of Wisconsin inbred strains\(^{6-8}\)) weighing 250 to 350 g maintained ad libitum on water and a standard pellet diet containing 0.4% NaCl since weaning in the animal resource center of the Medical College of Wisconsin. All of the protocols were approved by the institutional animal care committee.

Solutions

Hanks’ balanced salt solution was purchased from Invitrogen. Na\(^-\)-free solution was prepared by adding ChCl (154 mmol/L to distilled deionized H\(_2\)O). HEPES (20 mmol/L; Sigma Co) was added to all of the solutions and the pH adjusted to 7.40. Apocynin, N-methylamiloride, ChCl, nigericin, KR32568, NH\(_4\)Cl, and BaCl\(_2\) were purchased from Sigma Co. S3226 and cariporide were generously provided by Sanofi-Aventis Deutschland GmbH. Dihydroethidium (DHE) and 2',7'-bis-(2-carboxyethyl)-5-(and)-6-carboxyfluorescein, acetoxymethyl ester (BCECF) were purchased from Molecular Probes.

Determination of mTAL O\(_2\)^{−} Production and pH

Rats were anesthetized with sodium pentobarbital (60 mg/kg IP) and isolation of mTAL tissue strips performed as described previously.\(^{8}\) Thin tissue strips containing mTALs were placed on a glass coverslip coated with the tissue adhesive Cell-Tak (BD Biosciences) for fluorescence imaging. Tissue strips containing mTALs were loaded with either DHE (50 mmol/L) or BCECF (6 mmol/L) in Hanks’ balanced salt solution for 1 hour at room temperature. Loading buffer was then replaced with Hanks’ balanced salt solution and tissues were rested for an additional 15 minutes before being imaged. Coverslips were placed on a heated imaging chamber maintained at 37°C (Wanner Instruments) that allowed the rapid exchange of superfusion buffer and mounted on the stage of an inverted microscope.

Fluorescence measurements were made using a Nikon TE2000 inverted microscope with a ×60 water immersion (numeric aperture 1.2) objective lens. The signal was detected using a high-resolution digital camera (Photometrics Cascade 512B, Roper Scientific). Excitation was provided by a Sutter DG-4 175W xenon arc lamp (Sutter Instruments) that allowed high-speed excitation wavelength switching. Five to ten mTAL epithelial cells were selected within each tissue strip to quantify changes in fluorescent intensity of dyes using Metafluor imaging software (Universal Imaging). BCECF was excited at 440/10 and 490/10 nm. A 510/40-nm band pass emission filter was used to collect a BCECF fluorescent signal at 3-second intervals. Intracellular pH (pHi) was calibrated in situ at the end of each experiment using a 2-point calibration curve by exchanging the bath solution with saline solution containing nigericin (10 mmol/L) and KCl (140 mmol/L) of known pH.\(^9\) Because of the overlap in excitation and emission wavelengths of BCECF and DHE, O\(_2\)^{−} responses were determined in separate mTALs. A 445/40-nm and a 605/55-nm band pass emission filter were used to collect DHE (380/40X-445/40E) and ethidium (Eth; 480/40X-605/55E) signals. A Lambda-10-3 and rapid filter wheel changer (Sutter Instruments) was used to collect emission signals from DHE and Eth at 3-second intervals. Background Eth and DHE fluorescent signals were subtracted from the average intensity at all of the regions of interest containing mTAL epithelial cells. DHE and Eth signals were then normalized so that the ratio Eth:DHE at time 0 was equal to 1. The change in the ratio of Eth:DHE fluorescent signal across the duration of the experiment was then used as an index of O\(_2\)^{−} production.

NaCl Stimulation of mTAL O\(_2\)^{−} Production

O\(_2\)^{−} production was stimulated in mTALs of SS and SS.13\(^{BN}\) rats by increasing bath NaCl concentration through 154 to 254 and 500 mmol/L, for 200 seconds at each increment, over a 600-second period, as reported previously.\(^{10}\) O\(_2\)^{−} responses to incrementing the bath NaCl concentration were determined in response to incrementing the bath NaCl in the presence of KR32568 ([5-(2-methyl-5-fluorophenyl)furan-2-ylcarbonyl]guanidine [100 mmol/L], a selective inhibitor of NHE-1 (IC\(_{50}\) 0.23 μmol/L)\(^{11}\) and dual inhibition by cariporide (100 μmol/L) and S3226 (100 μmol/L), which are selective inhibitors of NHE-1 (IC\(_{50}\) 0.033 μmol/L)\(^{11}\) and NHE-3 (IC\(_{50}\) 0.23 μmol/L),\(^{12}\) respectively.

NH\(_4\)Cl Prepulse

To identify the source of amiloride-sensitive O\(_2\)^{−} production in mTALs of SS and SS.13\(^{BN}\) rats, we stimulated H\(^+\) efflux in mTAL epithelial cells under a number of conditions using the NH\(_4\)Cl prepulse method. In brief, this method involved adding 20 mmol/L of NH\(_4\)Cl solution to a bath containing tissue strips. Because mTALs are highly permeable to NH\(_4\)^{+}, but not NH\(_3\)^{+}, and this solution contains NH\(_3\)^{+} and NH\(_4\)^{+} in equilibrium, NH\(_4\)Cl preferentially enters the mTAL epithelial cells, resulting in rapid intracellular alkalinization (Figure 2). mTALs were left to bathe in this solution for 5 to 10 minutes, at which time pHi returned toward baseline levels. Once pHi reached a stable plateau, the NH\(_4\)Cl solution was quickly replaced with a vehicle solution that results in rapid acidification of the cell. The rate of recovery of pHi toward baseline levels and the production of O\(_2\)^{−} after acidification were then recorded.

Eight protocols were performed in total, four in which pH responses to an NH\(_4\)Cl prepulse were recorded in mTALs loaded with BCECF and four identical protocols in which O\(_2\)^{−} responses were recorded in mTALs loaded with DHE. Responses in mTALs from SS and SS.13\(^{BN}\) rats were compared within each protocol. In one group, an NH\(_4\)Cl prepulse was performed in bicarbonate-free saline (154 mmol/L) to stimulate NHE in mTALs. In a second group, an NH\(_4\)Cl prepulse was performed in the same medium with the exception that NaCl was replaced with ChCl to produce Na\(^-\)-free media to inhibit NHE activity. In a third group, an NH\(_4\)Cl prepulse was performed in Na\(^-\)-free media in the presence of BaCl\(_2\) (10 mmol/L). BaCl\(_2\) was added because this ion has been demonstrated previously to inhibit amiloride-insensitive H\(^+\) flux in mTALs.\(^{13}\) The final group contained the same medium as the third group with the addition of 100 μmol/L of N-methyl-amiloride. In some mTALs, apocynin (100 μmol/L) was added to the bath 30 minutes before stimulation by NH\(_4\)Cl prepulse in Na\(^-\)-free BaCl\(_2\) (10 mmol/L) media to determine the contribution of NAD(P)H oxidase.

Angiotensin II Stimulation of O\(_2\)^{−} Production

O\(_2\)^{−} production was stimulated in mTALs of SS and SS.13\(^{BN}\) rats by the addition of angiotensin II (1 μmol/L) to the bath, as reported previously.\(^{14}\) O\(_2\)^{−} responses were determined as the change in the ratio of Eth:DHE 200 seconds after the administration of angiotensin II. To determine whether O\(_2\)^{−} responses to angiotensin II may be mediated by amiloride-sensitive pathways, O\(_2\)^{−} responses to angiotensin II were also determined in both SS and SS.13\(^{BN}\) rats in the presence of N-methyl-amiloride (100 μmol/L).
significance was evaluated using an unpaired Bonferroni posthoc test. For all of the other data, significance was

\[ /H9262 \text{tor (100)} /H11006 \text{mol/L increased O}_2^-/H18528\text{ production.} \]

**Figure 1.** Effect of specific inhibition of NHE isoforms on NaCl-induced O\(_2^-\) production in mTALs of SS and SS.13\(^{BN}\) rats in response to incrementing extracellular NaCl concentration.

**Data and Statistical Analysis**

Data are expressed as mean ± SE. Responses in mTALs of SS and SS.13\(^{BN}\) rats were compared with a 2-way ANOVA using a Bonferroni post hoc test. For all of the other data, significance was evaluated using an unpaired t test. The level required to reach significance was \( P < 0.05 \).

For further methods, please see the online data supplement at http://hyper.ahajournals.org.

**Results**

**NHE Inhibitors on O\(_2^-\) Response to Incrementing Bath NaCl**

Incrementing bath NaCl concentration through 154 to 254 and 500 mmol/L increased O\(_2^-\) production in mTALs of both SS and SS.13\(^{BN}\) rats. In the presence of KR32568, a selective and potent inhibitor of NHE-1,\(^{10}\) total O\(_2^-\) production over the 600-second protocol was \( \approx 40\% \) greater in mTALs of SS rats compared with the responses observed in mTALs of SS.13\(^{BN}\) rats (Figure 1; \( P < 0.005 \)). Unlike N-methylamiloride administration, which we have reported previously to inhibit O\(_2^-\) production and abolished strain differences between mTALs of SS and SS.13\(^{BN}\) rats,\(^1\) our current data indicate that KR32568 did not reduce O\(_2^-\) responses in SS rats to the levels observed in mTALs of SS.13\(^{BN}\) animals. A similar response was observed in response to incrementing bath NaCl in the presence of cariporide and S3226, which are potent inhibitors of NHE-1 (IC\(_{50}\): 0.033 \( \mu \)mol/L\(^{11}\) and NHE-3,\(^{12}\) respectively. In the presence of both cariporide and S3226, O\(_2^-\) responses to incrementing bath NaCl remained elevated in mTALs of SS rats compared with mTALs of SS.13\(^{BN}\) rats (Figure 1; \( P < 0.005 \)). Neither outer medullary protein expression of NHE-1 nor NHE-3 was different between SS and SS.13\(^{BN}\) rats (please see the online data supplement).

**NH\(_4\)Cl Prepulse**

Baseline pH\(_i\) levels in mTALs of SS and SS.13\(^{BN}\) rats in each bath solution, as well as the rates of pH\(_i\) recovery over the first 20 seconds after cellular acidification by NH\(_4\)Cl prepulse, are given in the Table. Figure 2 demonstrates pH\(_i\) and O\(_2^-\) responses during the NH\(_4\)Cl prepulse in Na\(^+\)-free media with BaCl\(_2\). Note that O\(_2^-\) production was only stimulated during \( H^+ \) efflux after removal of NH\(_4\)Cl and not during \( H^+ \) influx.

As observed in Figure 3, in Na\(^+\)-replete media (154 mmol/L of NaCl; pH 7.40), pH\(_i\) recovered rapidly in both mTALs from SS and SS.13\(^{BN}\) rats after removal of NH\(_4\)Cl from the bath and cellular acidification. In the absence of bicarbonate, which inhibits Na\(^+\)/HCO\(_3^-\) exchange, the initial rate of pH\(_i\) recovery in Na\(^+\)-replete media can be used as an index of NHE activity.\(^9\) The initial rate of recovery of pH\(_i\) in Na\(^+\)-replete media was not different between mTALs of SS and SS.13\(^{BN}\) rats (Figure 3B). Despite activation of NHE and rapid recovery of pH\(_i\), no significant O\(_2^-\) produc-

**Table. pH\(_i\) in SS and SS.13\(^{BN}\) mTALs During NH\(_4\)Cl Prepulse**

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline pH(_i)</th>
<th>pH(_i) Recovery (Maximum Rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SS</td>
<td>SS.13(^{BN})</td>
</tr>
<tr>
<td>154 mmol/L of Na(^+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>7.32±0.04</td>
<td>7.23±0.03</td>
</tr>
<tr>
<td>0 Na(^+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>7.23±0.07</td>
<td>7.25±0.08</td>
</tr>
<tr>
<td>0 Na(^+) + BaCl(_2) (10 mmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>7.04±0.05</td>
<td>7.03±0.04</td>
</tr>
<tr>
<td>0 Na(^+) + BaCl(_2) (10 mmol/L) + NMA (100 ( \mu )mol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>7.08±0.06</td>
<td>7.03±0.04</td>
</tr>
</tbody>
</table>

Data show the baseline pH\(_i\), and maximal rate of recovery from cellular acidification in SS and SS.13\(^{BN}\) mTALs. Left, bath medium; baseline pH\(_i\), average pH\(_i\) over 60 seconds before removal of NH\(_4\)Cl from bath; pH\(_i\) recovery (maximum rate), slope of recovery of pH\(_i\) over 20 seconds from maximal acidification (pH/second); \( P \), result of unpaired t test comparing data obtained from SS and SS.13\(^{BN}\) mTALs. NS indicates not significant.

\(* P \) value is significant.
tion was observed in response to an NH4Cl prepulse in Na+-replete media in mTALs from either SS or SS.13BN rats (Figure 3A).

In Na+-free media in which NaCl was replaced with ChCl to inhibit NHE, pH recovery from an NH4Cl prepulse was less than that observed in Na+-replete media (Table). The rate of pH recovery, however, did not differ between mTALs from SS and SS.13BN rats (Figure 3D). In Na+-free media, O2− responses were observed in SS and SS.13BN rats corresponding with the time in which H+ efflux was occurring. These O2− responses did not differ, however, between mTALs from SS and SS.13BN rats (Figure 3C).

In Na+-free media in which BaCl2 had been added as a nonspecific inhibitor of ion transporters,15 pH recovery after cellular acidification was reduced compared with Na+-free media alone (Table). Importantly, the rate of recovery of pH in mTALs of SS rats was greater than that observed in mTALs of SS.13BN rats under these conditions (Figure 3F; P<0.05). In addition, O2− production associated with H+ efflux was significantly greater in mTALs of SS rats compared with mTALs of SS.13BN rats during this period (Figure 3E; P<0.05). The addition of N-methyl-amiloride reduced the rate of pH recovery in mTALs of SS rats to similar levels to those observed in mTALs of SS.13BN rats (Figure 3H) and completely abolished O2− production in response to recovery from cellular acidification (Figure 3G).

Role of NAD(P)H Oxidase

The NAD(P)H oxidase inhibitor apocynin reduced O2− production in SS mTALs in response to an NH4Cl prepulse in Na+-free media containing BaCl2 (Figure 4). The addition of angiotensin II (1 µmol/L), a well-known stimulator of NAD(P)H oxidase, stimulated the production of O2− in mTALs from both SS and SS.13BN rats. Importantly, in

Figure 2. Representative example of NH4Cl prepulse experiment in mTALs measuring pH and O2− production in Na+-free BaCl2 media. x axis, time (seconds); y axis (left) O2− production assessed as the ratio of Eth: DHE fluorescents (arbitrary units), (right) pHi dotted gray line, representative trace detailing pHi over the course of a single NH4Cl prepulse; solid black line, representative trace detailing O2− production over the course of a single NH4Cl prepulse (note that pHi and O2− data were obtained from separate experiments).

Figure 3. pH recovery and O2− production in mTALs of SS and SS.13BN rats after acidification by NH4Cl prepulse. A and B, Medium containing 154 mmol/L Na+; C and D, 0 Na+; E and F, 0 Na+ + BaCl2 (100 mmol/L); G and H, 0 Na+ + BaCl2 (100 mmol/L) + N-methyl-amiloride (100 µmol/L). Top A through G, O2− response to NH4Cl prepulse. Bottom B through H, pH recovery response to NH4Cl prepulse. x axis, time (seconds); time=0, point of maximal acidification after removal of NH4Cl from the bath. y axis (top), O2− production assessed as the ratio of ∆Eth:∆DHE fluorescents (arbitrary units), y axis (bottom), pHi; solid black lines represent mTALs from SS rats; solid gray lines represent mTALs from SS.13BN rats; data are mean±SE. *p<0.05 Tukey posthoc test comparing SS and SS.13BN responses; #p<0.05 for O2− response to NH4Cl prepulse; n=x/y number of observations (n) from mTALs of SS and SS.13BN rats, respectively.
Discussion
The major findings of this study are that N-methyl-amiloride inhibits $O_2^-$ production in mTALs by inhibiting $H^+$ efflux, specifically through a novel, Na+-insensitive $H^+$ transport pathway, not its classical target NHE, and that this amiloride-sensitive $H^+$ transport pathway is enhanced in SS rats. Our finding that a novel $H^+$ transport pathway mediates $O_2^-$ production in renal tubular cells is of particular relevance given the importance of $O_2^-$ and oxidant stress in cardiovascular and renal disease and represents a significant step forward in our understanding of free radical biology in the kidney. The observed upregulation of this oxidant producing pathway in one of the most commonly used models of hypertension, the Dahl SS rat, indicates that this pathway may contribute to the increased outer medullary oxidative stress and hypertension found in SS animals. In addition, our data suggest that amiloride analogues, eg, N-methyl-amiloride, may be useful inhibitors of this pathway and, therefore, of clinical relevance in diseases where oxidant stress is implicated, eg, salt-sensitive hypertension.

Sodium Stimulation of $O_2^-$ in the Presence of Specific NHE Inhibitors
In a previous study, we demonstrated that $O_2^-$ production in mTALs in response to cell shrinkage after incrementing bath NaCl was greater in mTALs of SS rats than that in mTALs of SS.13BN rats. This finding was important, because we have demonstrated previously that enhanced outer medullary oxidative stress contributes to the development of salt-sensitive hypertension in SS rats. Importantly, in our previous study, N-methyl-amiloride reduced (mTAL) $O_2^-$ production in SS rats, whereby $O_2^-$ production became equal in SS and salt-resistant control SS.13BN rats in response to cell shrinkage. We were, therefore, surprised in the current study when we found that, in the presence of the potent NHE-1 inhibitor KR32568, the rate of $O_2^-$ production remained elevated in SS rats compared with SS.13BN rats in response to incrementing bath NaCl from 154 to 254 to 500 mmol/L, as we had done in our previous study. Even the simultaneous administration of both cariporide and S3226 to inhibit both NHE-1 and NHE-3 activity failed to reduce $O_2^-$ production in mTALs of SS rats, which remained elevated above that in SS.13BN rats. Cariporide can inhibit both NHE-2 (IC$_{50}$: 1.6 $\mu$mol/L) and NHE-1, so dual pharmacological inhibition using cariporide and S3226 would have inhibited NHE-1, NHE-2, and NHE-3. Because these are the primary isoforms of NHE identified in mTALs, our data strongly indicated that NHE was not involved in the $O_2^-$ responses that we observed. In light of these data, as well as evidence that $H^+$ efflux stimulates mTAL NAD(P)H oxidase, we hypothesized that N-methyl-amiloride was inhibiting $O_2^-$ production in SS mTALs by blocking an $H^+$ transport pathway other than NHE.

NH$_4$Cl Prepulse Studies
To determine whether N-methyl-amiloride may be inhibiting $O_2^-$ production in SS mTALs by inactivating transport pathways other than NHE, we used the NH$_4$Cl prepulse method to activate $H^+$ efflux in SS and SS.13BN rats under a variety of bath conditions. Although there are numerous transporters present in mTALs capable of extruding $H^+$, NHE is the most effective at rapidly removing $H^+$ and dominates...
the pH recovery response after acidification. The rationale for inhibiting NHE in the current study was based on the idea that, because NHE dominates H⁺ extrusion, activation of less sensitive H⁺ transporters in response to an acid load would be limited and obscured by the ability of NHE to rapidly remove the stimuli. By inhibiting NHE using Na⁺-free media, the task of removing the H⁺ from the cell is then left to secondary, Na⁺-independent transport pathways. In this case, although the rate of overall H⁺ efflux would be slowed, more H⁺ would have to be extruded through these secondary pathways, because NHE is inactive. Given that O₂⁻⁻ production in response to an NH₄Cl prepulse was enhanced by inhibition of NHE, our data demonstrate that H⁺ efflux, through a secondary, Na⁺-independent H⁺ pathway, was responsible for H⁺ efflux–induced production of O₂⁻⁻. Only when NHE activity was blocked by reducing media [Na⁺] to 0 did cellular acidification stimulate significant O₂⁻⁻ production. Neither the rate of O₂⁻⁻ production nor the rate of recovery of pHi, however, was different between mTALs of SS or SS.13BN rats in Na⁺-free media unless BaCl₂ (10 mmol/L) was added to the bath. BaCl₂ was used in this study to further inhibit H⁺ transport not associated with amiloride-sensitive O₂⁻⁻ production thereby isolating and stimulating O₂⁻⁻ production further. We speculated that Ba²⁺ would inhibit unrelated but not amiloride-sensitive pathways of H⁺ efflux, because Ba²⁺ has been demonstrated previously to inhibit amiloride-insensitive H⁺ influx in mTALs but not amiloride-sensitive H⁺ influx in Na⁺-free media.13

In Na⁺-free media in the presence of BaCl₂, pHᵢ recovery was significantly greater in mTALs of SS rats compared with SS.13BN rats. Under these conditions, in response to cellular acidification using an NH₄Cl prepulse, O₂⁻⁻ production was also significantly greater in mTALs of SS rats compared with mTALs of SS.13BN rats. Importantly, the addition of N-methyl-amiloride significantly reduced the rate of pHᵢ recovery in mTALs of SS rats to levels observed in SS.13BN rats. In addition, N-methyl-amiloride completely abolished O₂⁻⁻ production in the Na⁺-free BaCl₂ media in response to an NH₄Cl prepulse. These data indicate that an amiloride-sensitive H⁺ transport pathway, other than NHE, is present in mTALs and that, when activated, this transport pathway mediates the production of O₂⁻⁻. In addition, our data indicate that this pathway is enhanced in mTALs of SS rats compared with mTALs of salt-resistant SS.13BN rats.

We were unable to detect N-methyl-amiloride–sensitive H⁺ transport in SS.13BN rats in the current study, suggesting that the pathway(s) identified in SS mTALs may not be active in SS.13BN mTALs. Opposing this conclusion, in Na⁺-free media containing Ba²⁺, an NH₄Cl prepulse stimulated significant O₂⁻⁻ production in mTALs of SS.13BN rats, and this could be abolished by the addition of N-methyl-amiloride (Figure 3E and 3G). Given these data, we conclude that it is likely that the pathway(s) detected in SS mTALs are also present in mTALs of SS.13BN rats. It would appear, however, that these pathways are less active in mTALs of SS.13BN rats and that the level of Na⁺-independent, amiloride-sensitive H⁺ transport in this strain is below detectable limits using the BCECF dye method.

Because Ba²⁺ is thought to inhibit both Renal Outer Medullary Potassium channel and Na⁺/K⁺-ATPase-mediated transport,15,16 the addition of Ba²⁺ would likely have dissipated mTAL membrane potential. Importantly, Liu et al17 have demonstrated that depolarization of macula densa stimulates O₂⁻⁻ production, raising the possibility that Ba²⁺ may have stimulated O₂⁻⁻ production in our study by dissipating membrane potential. However, it should be noted that the O₂⁻⁻ responses observed in our study occurred only during H⁺ efflux and were inhibitable by amiloride, suggesting that H⁺ efflux through amiloride-sensitive channels rather than depolarization mediated the response.

Although O₂⁻⁻ production was observed in Na⁺-free media in the absence of BaCl₂ in response to cellular acidification, it remains unclear whether it was because of activation of Ba²⁺-insensitive transporters at a reduced rate or a distinct pathway of H⁺ transport also capable of stimulating O₂⁻⁻. Importantly, only when Ba²⁺ was present were we able to identify differential pHᵢ and O₂⁻⁻ between SS and SS.13BN mTALs, indicating that a select BaCl₂-insensitive subgroup of H⁺ transporters is likely responsible for differences in amiloride-sensitive O₂⁻⁻ production in mTALs of SS and SS.13BN rats.

Physiological Relevance of Amiloride-Sensitive O₂⁻⁻ Production in mTALs and Its Relation to Salt-Sensitive Hypertension

Like NHE-1, which is known to be stimulated by both intracellular acidification and cell shrinkage (independent of pH),5,18 it appears that the amiloride-sensitive H⁺ transport pathway that we have begun to characterize in this study can be activated by multiple stimuli. We have demonstrated that cellular shrinkage after increased extracellular NaCl concentration, angiotensin II, and cellular acidification in Na⁺-free media can all stimulate N-methyl-amiloride–sensitive O₂⁻⁻ production in mTALs. It is unlikely that cellular acidification would normally act as a primary stimul in order to activate the O₂⁻⁻ production in vivo given that Na⁺ is present and NHE is active. However, other stimuli, eg, cell shrinkage or angiotensin II, would be expected to stimulate amiloride-sensitive O₂⁻⁻ production in vivo in a variety of physiological and pathophysiological conditions.

Although the factors that activate this pathway in vivo remain unclear, importantly, Li et al4 have demonstrated in the in vivo kidney that >50% of outer-medullary oxidative stress is amiloride sensitive, suggesting that pathways such as those identified in the current study are active. Given these data, we speculate that differences in amiloride-sensitive H⁺ transport and O₂⁻⁻ production observed between mTALs of SS and SS.13BN rats in the current study may account for differences in outer-medullary O₂⁻⁻ levels observed between these rat strains in vivo.3,19

Our data indicating that, in the presence of Na⁺, cellular acidification did not stimulate mTAL O₂⁻⁻ production significantly is in contrast to the results of Li et al4 who reported that Na⁺ was required for O₂⁻⁻ production after an NH₄Cl prepulse. The major difference between the present study and that of Li et al4 is that we performed fluorescent measurements of O₂⁻⁻ (DHE) and pHᵢ (BCECF) separately in different
tissue strips to avoid the possibility of overlapping fluorescent signals confounding our results. Li et al report that they measured pHi and O$_2^-$ production in mTALs simultaneously by dual loading mTALs with BCECF and DHE.

**What Is the Amiloride-Sensitive H$^+$ Transporter That Mediates O$_2^-$ Production in mTALs?**

Numerous transporters are capable of extruding H$^+$ from mTAL epithelial cells. Although in the current study we have ruled out a role of NHE in mediating mTAL O$_2^-$ production, the identity of the amiloride-sensitive H$^+$ transporter mediating enhanced O$_2^-$ production in SS mTALs remains undetermined. Our data do, however, indicate that the amiloride-sensitive H$^+$ current is associated with NAD(P)H oxidase. Many of the subunits of NAD(P)H oxidase are upregulated in the renal medulla of prehypertensive SS rats. In the current study, we demonstrate that apocynin, an inhibitor of NAD(P)H oxidase, abolished O$_2^-$ responses in response to activation of this amiloride-sensitive H$^+$ current by NH$_4$Cl. Furthermore, angiotensin II, which is a well-known activator of NAD(P)H oxidase, stimulated O$_2^-$ production in SS mTALs, and this could be inhibited by amiloride.

The membrane-bound subunit of NAD(P)H oxidase, NOX2, has been shown to be associated with voltage-gated H$^+$ channels in immune cells, the role of which appears to be to extrude H$^+$ and act as a charge compensator for the electrogenic generation of O$_2^-$ by the oxidase. Interestingly, these voltage-gated H$^+$ channels share a number of traits with that of the amiloride-sensitive H$^+$ transport pathway identified in mTALs in the current study. Both can be activated by intracellular acidification, both are unidirectional (only extruding H$^+$), and both are relatively insensitive to BaCl$_2$. Additional studies will be required to determine the specific molecular target of amiloride in mTALs that mediates the inhibition of O$_2^-$ production.

**Perspectives**

Many of the traits of salt-sensitive hypertension, eg, early stage renal failure, which are so pervasive in blacks, are recapitulated in the SS rat. O$_2^-$ has been demonstrated to enhance Na$^+$ reabsorption by NKCC and Na$^+/H^+$ exchangers in mTALs, and excess Na$^+$ reabsorption by the mTAL has been implicated in the development of salt-sensitive hypertension in a number of human populations, including blacks. In the current study we characterized H$^+$ transport in the mTALs of salt-sensitive and salt-resistant rat strains and identified a novel target for amiloride that, when activated, stimulates the excess production of O$_2^-$ We concluded that H$^+$ efflux from mTAL cells that results in O$_2^-$ production occurs only through a specific, N-methylamiloride-sensitive transport pathway, not H$^+$ efflux via NHE, and that this novel H$^+$ efflux pathway is enhanced in mTALs of salt-sensitive rats. The inhibition of this oxidant producing H$^+$ transport pathway by amiloride analogues merits further research as a potential treatment for renal oxidative stress and salt-sensitive hypertension.

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**Disclosures**

None.

**References**

A Novel Amiloride-Sensitive H⁺ Transport Pathway Mediates Enhanced Superoxide Production in Thick Ascending Limb of Salt-Sensitive Rats, Not Na⁺/H⁺ Exchange
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TITLE: A NOVEL AMILORIDE SENSITIVE H⁺ TRANSPORT PATHWAY MEDIATES ENHANCED SUPEROXIDE PRODUCTION IN THICK ASCENDING LIMB OF SALT-SENSITIVE RATS, NOT Na⁺/H⁺ EXCHANGE

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RUNNING HEAD: Superoxide production in Dahl SS mTAL.

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**Western blots:**
Rats were anesthetized with sodium pentobarbital (60mg/Kg/i.p), the left kidney flushed with chilled HBSS and the outer medulla snap frozen in liquid nitrogen. Tissue was homogenized and approximately 30mg of protein extracted from the outer medulla for western blot. For the detection of NHE-1 the membrane was immunoblotted with primary antibody (mouse anti rat NHE1 IgG, BD transduction Laboratories, 611774) in 1: 500 dilution, then secondary antibody (HRP conjugated rabbit anti mouse IgG, Abcam ab6728). For the detection of NHE3, the membrane was blotted with primary antibody (mouse anti rat NHE3 IgG, BD transduction Laboratories, 611776, dilution 1:250) and the secondary antibody (HRP conjugated rabbit anti mouse IgG, Abcam ab6728, dilution 1:20000). For β-actin detection, the primary antibody used was mouse anti β-actin monoclonal antibody (Abcam ab6276, 1:20000 dilution) and the secondary antibody was HRP conjugated rabbit anti mouse IgG, (Abcam ab6728 1:20000 dilution). The densitometry values were normalized to the band of β-actin re-probed in the same sample.

**Control stimuli**
Diethyldithiocarbamic acid (DETC; 1 mmol/L; Sigma) to inhibit superoxide dismutase, and menadione sodium bisulfite (500mol/L; Sigma) to stimulate mitochondrial O$_2^-$ production, were added to serve as positive control stimuli to test for dye loading and cell viability. Strong positive control responses were observed in all coverslips tested indicating that none of the protocols utilized exhausted the DHE dye or adversely effected cell viability.
Figure S1. Western blot analysis of NHE protein expression in renal outer medullary homogenates from SS (n=6) and SS.13^{BN} (n=6) rats. Top (a), representative example of exposed film demonstrating protein expression of NHE-1 and control protein β-actin in outer medullary samples from SS and SS.13^{BN} rats; Bottom (b), representative example of exposed film demonstrating protein expression of NHE-3 and control protein β-actin in outer medullary samples from SS and SS.13^{BN} rats; x-axis, rat strain; y-axis, protein expression (AU - arbitrary units); data is mean±SE; filled bar/arrows, SS; open bar/arrows, SS.13^{BN}.