A Novel Variant of the β-Subunit of the Amiloride-Sensitive Sodium Channel in African Americans

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ABSTRACT
The amiloride-sensitive sodium channel is responsible for the rate-limiting step of sodium reabsorption in the distal renal tubule, and thus may play a key role in the maintenance of sodium balance and blood pressure. In this study, a genetic variant that results in a change of threonine to methionine at amino acid 594 (T594 M) in the carboxy-terminus of the β-subunit of the amiloride-sensitive sodium channel has been identified. This variant was present in 6.1% of African-American subjects (N = 231) but was not seen in Caucasians (N = 192). Whole cell voltage clamp of B-lymphocytes from individuals with the T594 M variant showed similar basal membrane slope conductance, compared with the wild-type but increased response to cAMP analog.

Key Words: Blacks, blood pressure, ion channels, patch clamp techniques, sodium balance

The regulation of sodium balance in mammals is a process that involves a number of sodium transporters, many of which are expressed in the kidney. Exact sodium balance is ultimately determined by the distal nephron. Because the amiloride-sensitive sodium channel is a key regulator of salt reabsorption in the nephron, we looked for mutations in the gene encoding the β-subunit of this channel in African Americans—a group known to have a higher prevalence of both hypertension and salt-sensitivity when compared with Caucasians (1−5). The channel is composed of at least three subunits: α, β, and γ (6,7). The human genes encoding all three subunits have been identified and cloned (8−11). Mutations in the carboxy-terminus of the β and γ subunits of this channel have been proposed to be the underlying cause of Liddle’s syndrome, a rare autosomal dominant form of hypertension (12,13). The β-subunit of the amiloride-sensitive sodium channel has been shown to be expressed in the renal collecting duct (14) and in B-lymphocytes (15,16), and the electrophysiological characteristics of the channel in these two tissues have been shown to be indistinguishable (17). Whole cell patch clamp studies in B-lymphocytes from individuals with Liddle’s syndrome (18) and expression of Liddle’s syndrome mutant channels in Xenopus oocytes (19) have shown increased channel activity. More recently, this increased sodium conductance has been correlated with an increased number of channels on the oocyte membrane (20).

We report a novel variant that results in a substitution of methionine for threonine at Position 594, in the carboxy-terminus of the β-subunit. This variant was detected in African Americans but not in Caucasians. Whole cell voltage clamping in B-lymphocytes show that the variant was associated with enhanced sodium conductance in response to cAMP analog.

METHODS
Subjects were recruited from the Hypertension Clinics of the University of Cincinnati and the Veterans Administration Hospital, as well as from the community at large. The study was approved by the Institutional Review Board of the University of Cincinnati.

African Americans

All subjects underwent a brief clinical evaluation and provided a blood sample for measurement of plasma electrolytes and DNA isolation. Subjects were seated for 5 min. after which blood pressure recordings were done in triplicate by using a mercury manometer with the appropriate cuff size based on the upper mid-arm circumference. Korotkoff Phase V was used to define the diastolic pressure.

Treated hypertensive subjects were included if they reported that the onset of hypertension was before the age of 60 and that they had been under continuous treatment with antihypertensive medication for the previous 6 months. In hypertensive subjects not on antihypertensive treatment, average diastolic blood pressure was confirmed to be ≥90 mm Hg on a second visit a week later. All hypertensive subjects met the following criteria: no reported history of secondary hypertension; serum potassium level ≥3.5 mEq/L (unless they were being treated with thiazide or loop diuret-
Amiloride-Sensitive Sodium Channel Variant

was had to precede the diagnosis of diabetes mellitus by at least 5 yr. Normotensive subjects were included if systolic pressure was <150 and diastolic pressure was <90 mm Hg, based on the average of three readings on a single visit while not being treated with antihypertensive medications.

Caucasians

To determine allele frequency of the T594 M variant in Caucasians, two populations were combined: (1) 120 unrelated grandparents from the Centre d'Étude du Polymorphisme Humain (CEPH) pedigree for whom hypertension status is unknown (21), and (2) 72 unrelated Caucasians from the Cincinnati area (30 normotensive, 32 hypertensive, and ten of indeterminate blood pressure status).

DNA Analysis

DNA was extracted from peripheral blood by using Puregene DNA isolation kit (Gentra Systems Inc, Minneapolis, MN). Single-strand conformational polymorphism analyses were performed by amplifying DNA fragments of the final exon of human amiloride-sensitive sodium channel β-subunit (β-ENaC) from genomic DNA. Oligonucleotide primers βEnAC1746 and βEnAC1940 were used in polymerase chain reactions, as previously described (12). Products were fractionated on 0.6× MDE (Mutation Detection Enhancement) gels (AT Biochem, Malvern, PA) and 0.8× TBE (Tris Borate EDTA) as electrophoresis buffer at room temperature for 12 to 14 h. After autoradiography, the variant band was cut from the gel, and the DNA was eluted and reamplified before being sequenced with an ABI automated DNA sequencer (ABI, Foster City, CA). In all cases, both strands of DNA were sequenced.

Aldosterone Profiling

Eight subjects with the T594 M variant, of which four were hypertensive, were randomly chosen for aldosterone profiling and electrophysiological evaluations. Eight African-American subjects without the T594 M variant (of whom three were hypertensive) were chosen as control subjects such that age, sex, and body mass index were matched between the two groups. This aldosterone profiling protocol has been used to classify individuals in Liddle's syndrome kindreds (22). All subjects collected urine overnight. At approximately 8 a.m. the next day, blood was collected after the subject sat upright for 30 min. All subjects continued to follow their usual diet and medication treatment. Serum and urine aldosterone levels were measured by RIA (Coat-A-Count; Diagnostic Product Corporation, Los Angeles, CA). Normal ranges for the upright position are 5 to 30 ng/dL for serum aldosterone and 2 to 14 μg/24 h for urine aldosterone. Although no normal range has been established for overnight 12-h collections, 12-h aldosterone excretion rates have been shown to correlate with 24-h collection excretion rate in adults (23). Plasma renin samples were determined by RIA (New England Nuclear, Boston, MA). The normal range for upright plasma renin with this assay is 0.3 to 3.0 ng/mL per h.

Electrophysiological Studies

The electrophysiological measurements on lymphocytes were conducted in a single-blind manner (presence or absence of the variant was not revealed to experimenter). The bath medium was RPMI 1640 containing low Ca²⁺ (0.54 mM) supplemented with 10 mM N-hydroxymethylpiperezine-N'-2-ethanesulfonic acid to buffer the pH. The perforated-patch method was used (24,25). The pipette was tip-filled with recording medium containing (in mM): KCl; 70; K₂SO₄, 30; NaCl, 12; EGTA, 0.5; MgCl₂, 1; HEPES, 20; glucose, 10 (pH was adjusted to 7.2 and osmolarity to 300 mosmol). The pipette was then back-filled with nystatin-containing recording solution (200 to 300 μg/mL) prepared from a fresh stock consisting of 50 mg/mL of nystatin sonicated in dimethyl sulfoxide. Resistances of the pipettes were between 12 to 15 Mohm.

The slope conductance was measured from the linear portion of the ramp current elicited by a voltage ramp from −150 to +50 mV over the duration of 1 s, using the software pClamp (Version 5.5, Axon Instruments, Foster City, CA). At least four ramps at 0.2 Hz were averaged for each measurement. After the control ramps were obtained (two to three measurements), 300 μM 8-CPT-cAMP (8-CPT 8-(4-chlorophenylthio) adenosine 3,5-cyclic monophosphate), a membrane-permeable analog of cAMP, was superfused onto the cell from a blunt-tipped pipette (5 to 10 μm) placed nearby, and two to three more measurements were performed. Current records were filtered at 5 kHz, digitized, and stored for analysis.

Statistical Analysis

Results are expressed as mean ± SD or 95% confidence intervals. Plasma renin levels, aldosterone levels, sodium and potassium excretion rates, and ratio of urine aldosterone to potassium were log-transformed before analysis of variance. To correlate the aldosterone profile results with the electrophysiologic data, the ratio of the 8-CPT-cAMP-stimulated to basal conductance was averaged for each subject and used as the dependent variable in an analysis of variance (Statistical Analysis System; SAS Institute, Cary, NC).

RESULTS

Identification of a Missense Mutation at Codon 594 of the Amiloride-Sensitive Sodium Channel β Subunit

Single-strand conformational polymorphism analysis of the 3' end of the gene showed a variant band in some individuals of African-American descent. Elution of this band and subsequent sequence analysis revealed a single nucleotide substitution (C-T) in the coding strand at amino acid position 594. Sequencing the opposite strand confirmed the initial observation. The normal allele was sequenced and found to be identical to that reported by McDonald et al. (10). These results are shown in Figure 1. All individuals with this variant were heterozygous at this locus.

The frequencies of the T594 M variant are shown in Table 1. The clinical characteristics of the African Americans with and without the T594 M variant are shown in Table 2. There were no statistically significant differences between African Americans with and without the T594 M.
Amiloride-Sensitive Sodium Conductance in Lymphocytes With and Without the T594 M Variant

Membrane slope conductance of isolated B-lymphocytes from individuals without the T594 M variant (wild-type) and B-lymphocytes harboring the T594 M variant were measured. Slope conductances before and in the presence of 8-CPT-cAMP from the two groups were then compared. The basal slope conductances for the two groups were similar: 0.43 ± 0.24 nS (N = 15) for wild-type cells and 0.45 ± 0.21 nS (N = 12) for T594 M variant cells (P = 0.79). In the presence of 8-CPT-cAMP, slope conductance of responsive cells was increased to 1.20 ± 0.72 nS for the wild-type cells (paired t test, P < 0.05) and 1.99 ± 1.22 nS for the variant cells (paired t test, P < 0.05). One-way analysis of variance shows that the 8-CPT-cAMP-stimulated slope conductance increase in the T594 M variant is significantly higher than that for the wild-type (P < 0.05). Representative traces of the recordings obtained are shown in Figure 2. Because the amiloride-sensitive sodium channel is most active at very negative membrane potentials (more negative than −120 mV), the presence of channel activities obscured the measurements of the slope conductance at the negative potentials (see Figure 2). Thus, the slope conductance that we measured during 8CPT-cAMP application is likely to be an underestimation of the actual changes that had occurred.

The change in slope conductance in response to 8-CPT-cAMP measured for the variant could result from a higher number of amiloride-sensitive sodium channels (also an increase in probability of channel opening, unitary conductance, etc.) present in the variant lymphocytes or from a change in responsiveness of the channel to 8-CPT-cAMP. To compare the responsiveness of the amiloride-sensitive sodium channel with 8-CPT-cAMP in the wild-type and the variant, and to rule out the possibility that the response is related to the basal conductance, we examined the ratio of the 8-CPT-cAMP response to basal slope conductance in the same cell. The response ratio was 2.97 ± 1.13 (N = 15) for the wild-type. This ratio is in good agreement with the ratio we derived (3.12) from the data reported for normal B-lymphoid cells by Oh et al. (15). For the variant, the response ratio was 4.72 ± 1.94 (N = 12), which is significantly higher when compared with the wild type (P = 0.007). These results indicate that the T594 M variant is more responsive to 8-CPT-cAMP.

When amiloride (2 μM) was coapplied with 8CPT-cAMP, the slope conductance were 0.36 ± 0.24 nS (N = 10; control subjects without amiloride 0.39 ± 0.23 nS) and 0.57 ± 0.30 nS (N = 7; control subjects without amiloride, 0.55 ± 0.26) for wild-type and T594 M variant, respectively (Figure 2). The result showed that the enhancement in slope conductance by 8-CPT-cAMP occurs via an amiloride-sensitive conductance.

### Table 1. T594M variant in populations of unrelated subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>N T594M/N Tested</th>
<th>Percentage^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>African-American Hypertensive Subjects</td>
<td>7/126</td>
<td>5.6</td>
</tr>
<tr>
<td>African-American Normotensive Subjects</td>
<td>7/105</td>
<td>6.7^b</td>
</tr>
<tr>
<td>Caucasian Subjects</td>
<td>0/192</td>
<td>0^c</td>
</tr>
</tbody>
</table>

^a χ² = 12.2, P = 0.002 for the entire table.
^b χ² = 0.12, P = 0.72 versus hypertensive subjects.
^c χ² = 12.0, P = 0.0005 versus African-American subjects (first two rows combined).
TABLE 2. Clinical characteristics of African-American subjects*  

<table>
<thead>
<tr>
<th>Group</th>
<th>Genotype</th>
<th>N (m/f)</th>
<th>Age (yr)</th>
<th>Age Dx (yr)</th>
<th>BMI (kg/m²)</th>
<th>SBP (mm Hg)</th>
<th>DBP (mm Hg)</th>
<th>Rx (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertensive Subjects</td>
<td>Wild-type</td>
<td>119 (47/72)</td>
<td>52 ± 11</td>
<td>39 ± 12</td>
<td>32.6 ± 7.9</td>
<td>144 ± 22</td>
<td>92 ± 14</td>
<td>87</td>
</tr>
<tr>
<td>Hypertensive Subjects</td>
<td>T594M</td>
<td>7 (5/2)</td>
<td>62 ± 15</td>
<td>44 ± 16</td>
<td>28.4 ± 3.8</td>
<td>149 ± 18</td>
<td>91 ± 6</td>
<td>87</td>
</tr>
<tr>
<td>Normotensive Subjects</td>
<td>Wild-type</td>
<td>98 (30/68)</td>
<td>57 ± 17</td>
<td>n/a</td>
<td>28.1 ± 6.9</td>
<td>124 ± 14</td>
<td>77 ± 7</td>
<td>0</td>
</tr>
<tr>
<td>Normotensive Subjects</td>
<td>T594M</td>
<td>7 (3/4)</td>
<td>55 ± 14</td>
<td>n/a</td>
<td>27.2 ± 4.2</td>
<td>125 ± 10</td>
<td>77 ± 8</td>
<td>0</td>
</tr>
</tbody>
</table>

* Values shown are mean ± SD. N: number of subjects; m/f: male/female; Age Dx: age of diagnosis of hypertension; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; Rx: subjects on antihypertensive medications.

Aldosterone Profiles of Subjects With and Without T594 M

The aldosterone profiles of the 16 subjects whose lymphocytes were studied electrophysiologically are shown in Table 3. Although the serum aldosterone and urine aldosterone levels were statistically higher in the subjects with the T594 M variant, there was no statistical difference when the urine aldosterone level was normalized for potassium excretion. The levels of circulating aldosterone in individuals with the T594 M variant were of particular interest because earlier studies in mammalian nephrons show that aldosterone increases the Na+ conductance in the apical membrane through the activation of amiloride-sensitive sodium channel (26,27). To assess whether the higher aldosterone levels observed in individuals with the T594 M variant could account for the higher 8-CPT-cAMP-stimulated slope conductance in the lymphocytes from these individuals, an analysis of variance was performed using the ratio of 8-CPT-cAMP-stimulated-to-basal slope conductance as the dependent variable. The covariates urine sodium excretion, urine potassium excretion, and aldosterone excretion did not abolish the relationship between presence of the variant and higher 8-CPT-cAMP-stimulated slope conductance (P = 0.001).

DISCUSSION

We have identified a missense mutation T594 M in the carboxy terminus of the β-subunit of the amiloride-sensitive sodium channel that is present in normotensive and hypertensive African Americans (6.1%) but not in Caucasians. Electrophysiological studies of the amiloride-sensitive sodium conductance in lymphocytes revealed that this variant is associated with an enhanced 8-CPT-cAMP-stimulated response when compared with wild-type (4.72- versus 2.97-fold, respectively).

There is evidence that mutations in the carboxy-terminus of the β subunit of the amiloride-sensitive sodium channel can lead to abnormal channel behavior, causing sodium retention and hypertension. Mutations causing a premature truncation of the last 43 to 75 amino acids of the carboxy-terminus of the amiloride-sensitive sodium channel β-subunit have been identified in Liddle’s syndrome, an autosomal dominant form of hypertension (12). The T594 M variant that we have identified is located within this region (47 amino acids from the carboxy-terminus).

Our electrophysiological and clinical data for the T594 M variant differ from that reported for Liddle’s syndrome. The amiloride-sensitive sodium channels of B-lymphoid cells from Liddle’s patients did not respond to 8-CPT-cAMP (18), whereas the response to 8-CPT-cAMP in the T594 M variant was enhanced when compared with the wild-type. This lack of response in Liddle’s syndrome has been attributed to increased inward baseline sodium current. Recently, it has been reported that Liddle’s syndrome truncation mutations in the carboxy terminus of the β-subunit result in an increased number of channels expressed in Xenopus laevis oocytes (19). Analysis of the carboxy-terminus revealed a conserved eight-amino-acid motif (PPPXXXL) that when mutated, resulted in an increased number of sodium channels expressed on the cell surface (20). Although we cannot rule out whether the enhanced responses induced by 8-CPT cAMP in the T594 M variant result from an increase in the channel density on the cell surface, the increase in response ratio suggests that the enhanced slope conductance is likely a result of a change in an intrinsic property of the channel. Thus it is possible that the physiological role played by the T594 M variant (which is not contained within the PPPXXXL motif) may be different from variants observed in Liddle’s syndrome. The lack of suppression of aldosterone in individuals with the T594 M variant and its presence in normotensive individuals (five of seven of whom were over the age of 50) also contrasts with Liddle’s syndrome patients, in whom aldosterone secretion is suppressed secondary to sodium retention and in whom hypertension usually develops during the second decade of life (22).

Other investigators have suggested that cAMP can either activate or inhibit the activity of the amiloride-sensitive sodium channel. Bubien et al., have shown in lymphocytes that cAMP usually activates the amiloride-sensitive sodium conductance but inhibits channel activity after exposure to pertussis toxin (28). Stutts et al., reported that coexpressing the cystic fibrosis transmembrane regulator with the rat renal amiloride-sensitive sodium channel suppressed cAMP-stimulated channel activity (29). Our studies indicate that lymphocytes expressing the T594 variant...
sodium channel not only show an enhanced response to cAMP but also show a greater susceptibility to opening at negative potentials on some occasions, possibly because of the loss of a suppressive effect. The threonine residue at position 594 in the β-subunit may be involved in the regulation of channel activity, as it is a potential target for phosphorylation by protein kinase C (PKC) (10). It has been shown, for example, that activation of PKC by addition of phorbol 12,13-dibutyrate or mezerein abolished amiloride-sensitive sodium channel activity (30). The substitution of methionine for threonine at this site may therefore cause the channel to become resistant to the negative-regulatory effect of PKC, and could explain the enhanced response to 8-CPT-cAMP seen in the

Figure 2. Current-Voltage (I-V) relation measured in lymphocytes from individuals without the variant (left panel, wild-type) and from individuals harboring the variant (right panel) under perforated-patch recording. I-V relation was elicited and the slope conductance obtained as described in the method section. Trace A in both panels shows the I-V relation under control conditions. Trace B in each panel shows the I-V relation in the presence of 8-CPT-cAMP. Trace C in both panels shows the I-V relation in the presence of both of 8-CPT-cAMP and amiloride (2 μM). Variants are representative tracings from three different individuals.

The threonine residue at position 594 in the α-subunit may be involved in the regulation of channel activity, as it is a potential target for phosphorylation by protein kinase C (PKC) (10). It has been shown, for example, that activation of PKC by addition of phorbol 12,13-dibutyrate or mezerein abolished amiloride-sensitive sodium channel activity (30). The substitution of methionine for threonine at this site may therefore cause the channel to become resistant to the negative-regulatory effect of PKC, and could explain the enhanced response to 8-CPT-cAMP seen in the

T594 variant. It is possible that the mechanism of regulation of channel activity involves at least two distinct sites—a positive modulatory site that increases channel activity and a negative modulatory site that decreases channel activity. The sites may be on the sodium channel itself or on its associated regulatory complex. Regulatory sites other than the cAMP and PKC sites, such as G protein binding sites, may also exist (28).

An important and yet unresolved question is whether this variant, apparently specific to African Americans, plays a role in the higher prevalence of salt-sensitive hypertension seen in this population. Although there was no significant difference in the prevalence of the T594 M variant between the hyper-
TABLE 3. Aldosterone profile of a subset of African-American subjects with and without T594M variant

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-Type (N = 8)</th>
<th>T594M Variant (N = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Potassium (mEq/L)</td>
<td>4.2 (3.9 to 4.6)</td>
<td>4.5 (4.3 to 4.7)</td>
</tr>
<tr>
<td>Plasma Renin Activity (ng/mL per h)</td>
<td>0.45 (0.19 to 1.04)</td>
<td>0.37 (0.17 to 0.78)</td>
</tr>
<tr>
<td>Serum Aldosterone (ng/dL)</td>
<td>6.9 (4.4 to 10.8)</td>
<td>13.3 (9.9 to 17.9)</td>
</tr>
<tr>
<td>Urine Sodium (mEq/12 h)</td>
<td>70 (55 to 89)</td>
<td>62 (40 to 62)</td>
</tr>
<tr>
<td>Urine Potassium (mEq/12 h)</td>
<td>21 (14 to 32)</td>
<td>27 (17 to 41)</td>
</tr>
<tr>
<td>Urine Aldosterone (µg/12 h)</td>
<td>2.0 (1.2 to 3.2)</td>
<td>4.4 (2.8 to 6.7)</td>
</tr>
<tr>
<td>Aldosterone:Potassium Ratio c</td>
<td>94 (66 to 135)</td>
<td>172 (92 to 325)</td>
</tr>
</tbody>
</table>

a Includes four hypertensive and four normotensive subjects in the T594M group, and three hypertensive and five normotensive subjects in the wild-type group. All values reported except potassium are the anti-log of the mean log-transformed value (95% confidence intervals).

b P = 0.03 by analysis of variance; all others are not significantly different.
c Nanograms of urine aldosterone per milliequivalent of urine potassium.

tensive and normotensive populations that we studied, this does not preclude it from being one of the contributing factors to the trait. The phenotypic contribution of the T594 M variant may not be evident in normotensive subjects who harbor the variant without the additive effects of other genetic or environmental factors.

It is important to point out some of the limitations in the interpretation of our observations. First, our electrophysiological studies were whole cell patch clamp measurements and not single channel recordings. Second, although it has been shown by reverse transcription-polymerase chain reaction that mRNA encoding the β-subunit is present in lymphocytes from human (15) and rat (16), the alpha subunit of this channel is probably a different isoform both in rat and human lymphocytes from the alpha subunit expressed in the renal collecting duct (15, 16).

Nonetheless, it has been shown that amiloride-sensitive sodium channel activity is increased in lymphocytes from individuals with Liddle’s syndrome (similar to what has been observed in the oocyte expression system of Liddle’s mutations). Therefore, although it is possible that the difference in whole cell electrophysiological activity conferred by the β-subunit variant T594 M in B-lymphocytes would also be present in the sodium channel expressed in the renal collecting tubule, this has not been proven in our study. If this variant does predispose to salt-sensitive hypertension, it would have to alter the function of the amiloride-sensitive sodium channel in the renal collecting duct.

Whether the T594 M variant confers any selective advantage to individuals who harbor the variant gene remains unknown. It is possible that the increased ability to reabsorb sodium conferred by the variant channel may be an advantage in certain conditions of salt deprivation, or, alternatively, increased protection against volume depletion in conditions of water scarcity.

The discovery of the T594 M genetic variant in the β-subunit of the amiloride-sensitive sodium channel, a key regulator of renal sodium handling, and the altered electrophysiological properties associated with it may be one of the first steps in establishing the amiloride-sensitive sodium channel as a genetic component of essential hypertension in African Americans.

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