Alpha-Adducin Polymorphism, Salt Sensitivity, Nitric Oxide Excretion, and Cardiovascular Risk Factors in Normotensive Hispanics

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**Background:** Genetic and environmental factors determine the blood pressure (BP) response to changes in salt intake. Mutations in the α-adducin gene may be associated with hypertension and salt-sensitive hypertension. We investigated whether one α-adducin polymorphism, the Gly460Trp (G/T) variant, was associated with salt sensitivity, nitric oxide (NO) production; and cardiovascular risk factors in healthy adult normotensive Venezuelans.

**Methods and Results:** Subjects (n = 126) were screened for salt sensitivity. The α-Adducin polymorphism was tested in salt-sensitive (SS) and salt-resistant (SR) subjects. The G/T and G/G (wild gene) groups had similar BP levels. The G/T subjects had higher LDL-cholesterol (P = .01) and postload glucose AUC (P = .03) than G/G individuals. Genotype frequencies were not associated with BP or salt sensitivity (G/G, 38.1% SS and 61.9% SR vs G/T, 40.7% SS and 59.3% SR). Shifting from high salt to low salt diet produced comparable reductions in systolic BP and diastolic BP in G/T and G/G groups. The G/G and G/T groups excreted similar amounts of sodium on high and low salt diets. The SR subjects carrying the wild or the mutated gene showed no changes in NO metabolite excretion at different levels of salt intake. In SS subjects, the level of NO metabolite excretion was highly dependent on salt intake. A combination of SS and 460Trp mutation enhanced the sodium-dependent modulation of NO production.

**Conclusions:** In normotensive Venezuelans, the α-adducin G/T polymorphism was not associated with BP, salt sensitivity, or with sodium excretion during sodium loading or restriction. G/T was associated with increased LDL-cholesterol and postload glucose levels. In SS, G/T was associated with greater salt-dependent modulation of NO excretion. However, this larger increase in NO excretion was not associated with a larger decrease in BP.

**Key Words:** α-Adducin polymorphism, nitric oxide, salt sensitivity, blood pressure, dysmetabolic cardiovascular syndrome.
(T/T) were SS and had low-renin hypertension. Because of the controversial findings, we further investigated the relationships between 460Trp polymorphism and salt in Hispanics, a previously not studied ethnic group.

Current evidence indicates that nitric oxide (NO) plays a role in intrarenal hemodynamics, sodium homeostasis, and in SS hypertension. Inhibition of NO synthesis induces salt sensitivity in rats, an effect reversed by administration of the NO precursor, l-arginine. Urinary excretion of NO is modulated by the intake of salt in SS, whereas it is independent of salt intake in salt-resistant (SR) animals and human subjects. In SS Dahl/Rapp rats and in SS humans, the urinary excretion of NO is markedly reduced when exposed to a high intake of salt. The role of genetic factors in the NO response to changes in salt intake has not been explored. In the present study we investigated whether the α-adducin 460Trp polymorphism determines the defective NO production reported in SS individuals when exposed to a high salt intake.

Cardiovascular risk factors, such as hypertension, left ventricular hypertrophy, and dyslipidemia, have been associated to α-adducin polymorphism. However, such associations have not been explored in Hispanic subjects. Consequently, in this study we also investigated the association between α-adducin polymorphism and cardiovascular risk factors.

In summary, the existence of an association between α-adducin 460Trp polymorphism and salt sensitivity, BP levels, cardiovascular risk factors, and salt-induced modulation of urinary NO excretion, was investigated in Hispanics, a previously not studied ethnic group.

Methods

Study Population

A total of 126 subjects attending our Center for the Detection and Treatment of Silent Cardiovascular Risk Factors were evaluated for salt sensitivity. Only SS and SR individuals were evaluated in this study. The exclusion criteria were: age more than 70 years; a history of angina pectoris, myocardial infarction, congestive heart failure, valvular heart disease, cerebral infarction or hemorrhage, transient ischemic attacks, arteriosclerosis obliterans, pulmonary disease; any patient with active disease, evidence of renal or hepatic dysfunction, urinary tract infection, active inflammatory disease states, severe hypertension, diabetes mellitus; treatment with organic nitrates, women on birth control pills, and serum creatinine concentration more than 2 mg/dL. Any medication was discontinued at least 4 weeks before the sodium-sensitivity protocol was started.

Determination of Salt Resistance and Salt Sensitivity

Patients were placed on a liberal sodium intake diet, prescribing in addition a total of 12 tablets a day, each containing 1 g (17.1 mmole) of sodium chloride. Subsequently, patients received a low salt diet (20 to 40 mmole/d) for 7 days. On days 6 or 7 of both sodium diets, patients returned to the center for the following procedures: systolic BP, diastolic BP, and heart rate (HR), 24-h sodium excretion, 24-h nitrate and nitrate excretion, serum and urinary creatinine levels. Blood pressure was measured with a standard mercury sphygmomanometer. Korotkoff sounds 1 and 5 were used to record systolic BP and diastolic BP, respectively. The BP measurements were done with the patient in the supine position after the patient had rested for at least 30 min. The average of at least three determinations was used. The mean BP was calculated as one-third of the pulse pressure added to the diastolic pressure. Patients were classified as salt sensitive and salt resistant. If the difference in mean BP between high and low sodium weeks was equal to or more than 10 mm Hg, the patient was deemed SS. Salt resistance was defined as increases of less than 3 mm Hg, no change, or decreases in mean BP.

Genetic Analysis

DNA was extracted from blood anticoagulated with EDTA according to standard protocols. The G to T substitution polymorphism resulting in the genetic variant of amino acid residue 460, located at nucleotide position 614 of exon 10 of the α-adducin gene (GenBank accession number L29294), was evaluated. Genotyping was performed with mutagenically separated polymerase chain reaction (MS-PCR). Briefly, two allele-specific primers of different lengths: FP-614G: 5'-GGGGCGACGAAAGCTTCCGAGGTAG-3'; FP-614T: 5'-GCTGAACTCTTGCCCAAGGGCAGCGAAGCCTCCGGAGATT-3', and their nonselective complementary strand primer: RP-614, 5'-CCTCCGAACCCCAAGCTACCCA-3', were used in a single-tube reaction assay. Additional base substitution at different mutagenic positions were introduced into the allele-specific primers, as was described elsewhere. This allowed clear separation between the two alleles during the subsequent amplifications steps by reduction of cross-reactions. The specificity of MS-PCR for the different genotypes was confirmed by direct sequencing of the amplified DNA (data not shown).

The PCR was performed on a Perkin Elmer Thermocycler in a 50-μL reaction mixture containing 2 μL of genomic DNA solution containing 0.23 μmol/L of FP-614G, 0.64 μmol/L of FP-614T, and 1.6 μmol/L of RP-614, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 25 μmol/L each dNTPs, 0.4 U Ampli-Taq DNA polymerase (Promega, Madison, WI), and 3 mmol/L MgCl2. The initial denaturation for 3 min at 95°C was followed by 45 cycles of denaturation for 20 sec at 94°C, annealing for 30 sec at 60°C and extension for 30 sec at 72°C. The size of PCR products was 220 bp and 234 bp for the 460Gly and the 460Trp alleles, respectively, which were clearly resolved on a 4% agarose gel (Promega). After staining of the gels with ethidium bromide, PCR products were visualized under ultraviolet light.
Subjects homozygous for the 460Gly or 460Trp alleles were identified as G/G or T/T, respectively. Subjects heterozygous for the 460Trp allele were identified as G/T.

**Determination of Nitrates and Nitrates in Urine Samples**

Nitrates plus nitrates were quantitated using a modification of a commercially available NO assay kit (Oxford Biomedical Research Inc., Oxford, MI). Three days before starting the high salt and low salt diets, and during the 7 days of the high and low salt diets, patients were asked to refrain from canned foods, black tea, meat and meat derivatives, and from processed food. Twenty-four-hour urine samples were collected at the end of the high and low sodium diets (10 days restriction from nitrate and nitrite containing foods). Urine was frozen at −60°C until required for assay. Urine was diluted 1:6 to 1:12 (vol: vol) with distilled water before the assay. After precipitation of the protein content, the nitrates present in the urine supernatant were reduced 1:1 to nitrates by incubation with metallic cadmium beads for 24 h. The total nitrite concentration was then estimated by the Griess reaction, using a multiwell microplate for reading of sample absorbance at 540 nm. This value represents the total amount of urine NO end products (nitrite + nitrate). Urine samples were processed in duplicates.

**Statistics and Data Analysis**

Categorical variables were compared by means of the χ² test. Continuous variables were compared by the Student t test for independent samples and paired t test for paired samples or by a one-way ANOVA followed by a Duncan’s test. Genetic equilibrium was confirmed by the Hardy-Weinberg criterion and was analyzed by the χ² test. Results are shown as mean values ± SEM; differences were considered significant at values of P < .05.

**Results**

A total of 126 consecutive subjects were tested for salt sensitivity. The average sodium excretion during the high and low salt diets was 305 ± 8 mEq/d and 37.8 ± 2 mEq/d, respectively. Of the 126 subjects, 35 (27.7%) were SS and 55 (43.7%) were SR (Table 1). The rest of subjects had intermediate salt sensitivity. The SS subjects had higher baseline systolic BP and diastolic BP than SR individuals (Table 1). No significant differences in fasting and postload glucose, cholesterol (total, LDL, and HDL), triglyceride, and urinary albumin excretion levels were encountered between SS and SR subjects (Table 1). The average reduction in systolic BP/diastolic BP induced by the shift from high salt to low salt diet was 16 ± 1.81 ± 1.3 mm Hg in SS and 0.0 ± 0.8 ± 0.6 mm Hg in SR subjects (Fig. 1). The baseline daily urinary excretion of sodium for all patients averaged 146 ± 8 mEq/d, and it was not different in SR (149 ± 8 mEq/d) and SS individuals (143 ± 10 mEq/d). During high salt and low salt diets the sodium excretion averaged 306 ± 14 and 40 ± 4 mEq/d, respectively, in SR, and 305 ± 13 and 34 ± 4 mEq/d, respectively, in SS individuals.

To determine the existence of an association between salt sensitivity and α-adducin polymorphism, genotyping with mutagenically separated polymerase chain reaction was performed in SS and SR individuals. The wild gene (G/G) was found more frequently (70% of subjects), irrespectively of whether the subjects were SS or SR. Nearly 30% of SS and SR individuals were heterozygous for the 460Trp mutation (G/T). No subject was found to be homozygous for the 460Trp allele (T/T) (Table 2). The sample examined was in Hardy-Weinberg equilibrium, and no subject T/T was found possibly because of the sample size.

The demographic characteristics of G/G and G/T groups are shown in Table 3. Compared to the G/G group, the G/T subjects had higher levels of LDL-cholesterol (P = .01) and higher postload AUC for glucose (P = .03) (Table 2). No significant difference was observed in the age, systolic BP, diastolic BP, fasting serum glucose, triglyceride, and urinary albumin excretion levels of both groups. The BP response to changes in salt intake was also evaluated in both groups (Fig. 1). Shifting from a high salt to a low salt diet produced comparable reductions in systolic BP and diastolic BP in G/G and G/T subjects (Table 4 and Fig. 1).

The urinary excretion of sodium is depicted in Table 4. High salt intake increased the urinary excretion of sodium and salt restriction lowered it. There was no significant difference in the amount of sodium excreted by G/G and G/T subjects when either on high or low sodium diets (Table 4). Similarly, no difference in the urinary excretion of potassium was observed between groups (data not shown).

![Table 1. Clinical characteristics of salt sensitive and salt resistant subjects](image-url)
The urinary excretion of NO metabolites (nitrates + nitrites) was dependent on the level of salt intake in SS, but not in SR subjects (Fig. 2). In SS, the urinary excretion of NO metabolites was reduced as the level of salt intake increased. Shifting from the high salt to the low salt diet led to a marked increase in the urinary excretion of NO metabolites in SS subjects. Interestingly, SS individuals carrying the 460Trp mutation (G/T) showed much greater salt dependence of NO metabolite excretion than the SS- G/G (Figs. 2 and 3). The SR carrying the wild or the mutated gene showed no significant change in NO metabolite excretion at different levels of salt intake (Figs. 2 and 3).

### Discussion

**α-Adducin and BP Levels**

Initial studies in hypertensive animals and humans indicated that the 460Trp mutation of the α-adducin gene was associated with the level of BP. However, other investigators have failed to show such an association. Ethnocity, clinical characteristics of subjects, and baseline BP levels played a role in determining the association. Genetic factors are not the only contributors to BP levels; lifestyle factors and environmental factors also play a significant role.

### Table 3. α-Adducin polymorphism: clinical characteristics of study subjects

<table>
<thead>
<tr>
<th>Subjects (n)</th>
<th>G/G</th>
<th>G/T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>40.1 ± 1.5</td>
<td>41.2 ± 2.3</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>76.5 ± 2.1</td>
<td>80.9 ± 3.1</td>
</tr>
<tr>
<td>WHR</td>
<td>0.89 ± 0.01</td>
<td>0.9 ± 0.01</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.3 ± 0.6</td>
<td>30.6 ± 1.09</td>
</tr>
<tr>
<td>FSG</td>
<td>92 ± 1.6</td>
<td>100 ± 4.7</td>
</tr>
<tr>
<td>AUC-G</td>
<td>57 ± 5</td>
<td>79 ± 8*</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>207 ± 6.1</td>
<td>228 ± 8.5</td>
</tr>
<tr>
<td>LDL-C</td>
<td>121 ± 7.2</td>
<td>153 ± 8.5†</td>
</tr>
<tr>
<td>HDL-C</td>
<td>42.1 ± 1.8</td>
<td>39.6 ± 2.3</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>151 ± 12.9</td>
<td>146 ± 15.4</td>
</tr>
<tr>
<td>UAE</td>
<td>9.0 ± 0.8</td>
<td>9.6 ± 1.5</td>
</tr>
</tbody>
</table>

FSG = fasting serum glucose in mg/dL; AUC-Glucose = area under the curve for glucose after a 75-g oral load of glucose in mg/dL/h. Total cholesterol, LDL-C, HDL-C, and triglycerides in mg/dL. UAE = urinary albumin excretion in mg/24 h.

* P < .05; † P < .01.

### Table 4. BP and electrolyte excretion during high and low salt intake in Gly460Gly and Gly460Trp subjects

<table>
<thead>
<tr>
<th>Subjects (n)</th>
<th>G/G</th>
<th>G/T</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNa⁺-SBP (mm Hg)</td>
<td>123.3 ± 2.2</td>
<td>122.9 ± 3.4</td>
</tr>
<tr>
<td>HNa⁺-DBP</td>
<td>79.6 ± 1.2</td>
<td>81.6 ± 2.5</td>
</tr>
<tr>
<td>UNa⁺/d</td>
<td>309.4 ± 11.2</td>
<td>297 ± 20.4</td>
</tr>
<tr>
<td>LNa⁺-SBP</td>
<td>116.4 ± 1.7</td>
<td>117 ± 2.2</td>
</tr>
<tr>
<td>LNa⁺-DBP</td>
<td>76.6 ± 1.1</td>
<td>79.1 ± 2.0</td>
</tr>
<tr>
<td>UNa⁺/d</td>
<td>38.4 ± 3.5</td>
<td>36.2 ± 6.0</td>
</tr>
<tr>
<td>Δ SBP (mm Hg)</td>
<td>6.8 ± 1.2</td>
<td>5.9 ± 1.8</td>
</tr>
<tr>
<td>Δ DBP (mm Hg)</td>
<td>3.2 ± 0.8</td>
<td>2.8 ± 1.4</td>
</tr>
<tr>
<td>Δ UNa⁺/d</td>
<td>287.1 ± 15.3</td>
<td>291.2 ± 26.4</td>
</tr>
</tbody>
</table>

UNa⁺ = urinary sodium excretion in mEq/d during a high sodium diet (HNa⁺-) and a low sodium diet (LNa⁺-).

Systolic and diastolic BP in mm Hg.

HNa⁺-SBP and DBP = BP during a high sodium diet.

LNa⁺-SBP and DBP = BP during a low sodium diet.

Δ SBP was calculated as SBP during high salt minus SBP during low salt.

Δ DBP was calculated as DBP during high salt minus DBP during low salt.

Δ UNa⁺/d was calculated as UNa⁺/d during high salt minus UNa⁺/d during low salt.
level are some of the factors determining the existence or lack of positive associations. To our knowledge no study has evaluated the relations between \( \alpha \)-adducin polymorphism and BP in Hispanics. Although this study was not designed to be a population study, as the polymorphism was only studied in SS and SR individuals (subjects with intermediate salt sensitivity were not genotyped), no association was observed between the \( \alpha \)-adducin Gly460Trp polymorphism and the individuals BP in normotensive Hispanics. Interestingly, previous studies in other normotensive ethnic groups have failed to show such an association (see Ref. 6 for review). It thus seems that the presence of the G/T mutation in the \( \alpha \)-adducin protein does not play a significant role in determining BP levels in normotensive individuals. It is possible that the polygenic nature of the population BP variation could hinder any phenotypic effect that the G/T mutation might exert.

\( \alpha \)-Adducin and Salt Sensitivity of BP

In an earlier study conducted in Italian hypertensives, it was shown that subjects carrying the 460Trp \( \alpha \)-adducin polymorphism experienced greater BP changes in response to acute salt volume loading and depletion, than those carrying the wild gene (G/G). However, in a subsequent study in hypertensives and in the present study in normotensives, no association between G/T polymorphism and salt sensitivity was observed. In the present study, the frequency of G/T and G/G alleles was similar in SR and SS subjects. In addition, shifting from high salt to low salt diets produced similar BP reductions in subjects with the mutated and the wild gene. The discrepancies observed between studies may be due to differences in ethnicity, in the clinical characteristic of study patients, the protocols and criteria used to study and classify SS and SR subjects, and in heterozygosity or homozygosity for the
460Trp allele. In the study by Ciechanowicz et al.\textsuperscript{13} and in the present study, subjects were either nonresponsive (SR) or highly responsive to changes in dietary salt (SS; $\geq 10$ mm Hg decrease in mean BP). Thus, the polymorphism was assessed in these two clearly separated groups. Cusi et al.\textsuperscript{9} did not classify hypertensives as SS and SR and thus did not report the frequency of the polymorphism in the two groups. They also used a rapid intravenous infusion of saline for sodium loading and a low salt diet combined with repeated furosemide doses for salt depletion.\textsuperscript{9} In addition, it was reported that the G/T allele was associated with larger BP decreases after chronic diuretic treatment.\textsuperscript{9,10} Consequently, the reported greater BP reductions observed in the G/T group, may in addition to salt sensitivity be due to an enhanced response to diuretics, which is associated with, but it is not exclusive, of salt sensitivity. Consequently, the discrepant results\textsuperscript{9,13} may be related to the fact that an enhanced BP response to diuretics does not necessarily equate to salt sensitivity as defined by the (high–low) salt diet protocol. A recent study showed that only hypertensives homozygous for the 460Trp polymorphism (T/T) ($n = 9$) had a greater systolic BP response to changes in salt intake.\textsuperscript{14} However, and as shown by Ciechanowicz et al.\textsuperscript{13} and in the present study, subjects heterozygous for the 460Trp allele (G/T) had similar BP response to dietary changes in salt intake than those carrying the wild gene (G/G).\textsuperscript{14} Unfortunately, the association between G/T and SS. In support of our findings, the G/T mutation was not related to exchangeable sodium, plasma volume, total body water, atrial natriuretic peptide, plasma renin activity aldosterone, cellular sodium, and transmembrane sodium efflux in young (off-springs 16 to 24 years) and older (parents 35 to 64 years) Scottish subjects.\textsuperscript{23} It remains to be seen whether T/T homozygosity is associated with salt sensitivity.

\section{α-Adducin Polymorphism and Cardiovascular Risk Factors}

In our study, the G/T group had significantly higher levels of LDL-cholesterol and a higher area under the curve for glucose during an oral glucose tolerance test, than the G/G subjects. An association between lipid levels and α-adducin polymorphism was recently described in familial combined hyperlipidemia.\textsuperscript{11} These researchers showed that the number of subjects carrying the 460Trp allele was significantly higher in patients with hyperlipidemia than in spouse controls.\textsuperscript{11} At present, genotype frequencies for α-adducin polymorphism in hyperlipidemia have not been determined. However, our results provide preliminary evidence to suggest that increases in LDL-cholesterol and early abnormalities in glucose tolerance may be associated with α-adducin polymorphism.

\section{α-Adducin Polymorphism and NO Metabolite Excretion}

Alterations in renal interstitial hydrostatic pressure and papillary hemodynamics contribute to the altered pressure–natriuresis relationship present in salt-sensitive subjects.\textsuperscript{26,27} It has been proposed that NO modulates intrarenal hemodynamics, sodium homeostasis, and plays a role in SS hypertension. Genetic defective NO synthesis or treatment with NO inhibitors induces salt sensitivity,\textsuperscript{17–19,28} whereas increased NO synthesis corrects salt sensitivity.\textsuperscript{15,19,29} High salt intake inhibits NO production in SS Dahl/Rapp rats.\textsuperscript{15} In SS human subjects, a high intake of salt decreases NO metabolite excretion; an effect reversed by a low salt diet.\textsuperscript{20} This original observation was confirmed in a larger patient population in the present study. In addition, we observed that G/T subjects had a stronger modulation of NO excretion by salt intake, with larger increases in NO production when the salt intake is reduced. The SR carrying the wild or the mutated gene had comparable levels of NO excretion at high salt and low salt dietary intakes (present study). In summary, in SS subjects a high intake of salt increases BP and decreases NO excretion. In SR, a high intake of salt neither affects BP nor NO excretion. In SS individuals, salt restriction lowers BP and increases NO production; whereas in SR, it fails to affect either BP or NO excretion. Our findings indicate the existence of a salt–BP modulation of NO production in SS. At this stage, we cannot determine whether salt-induced changes in NO production are the cause of SS or whether salt-induced changes in BP are responsible for the changes in NO excretion.\textsuperscript{15,20,30} An additional complicating factor is the origin of urinary NO; namely, it may reflect NO production at renal, systemic, or at both sites. If NO is responsible for SS, then salt restriction should have induced greater BP lowering in G/T SS than in G/G SS subjects. However, this was not the case. Salt restriction induced much greater increases in NO excretion in G/T SS than in G/G SS subjects, despite the fact that comparable BP reductions were observed in both groups. An additional, not yet determined mechanism, may counteract the effect of NO.
In a previous study, high salt diet was shown to reduce NO levels equally in SS and SR African American hypertensives. In our study, on the other hand, a high salt diet only reduced NO excretion in SS subjects. Differences in ethnicity (African Americans versus Hispanics) and in the levels of BP (hypertensives versus normotensives) may account for the discrepancies observed (see Ref. 20 for discussion). The α-Adducin gene polymorphism is an additional determinant of the magnitude of salt-induced changes in NO excretion, a factor not investigated in the study on African American hypertensives.

In conclusion, in healthy Venezuelan normotensive adults, there is no association between the α-adducin 460Trp mutation (G/T), and the state of salt sensitivity or the level of BP. The frequency of the heterozygous 460Trp allele was similar in SS and SR subjects. The α-adducin 460Trp mutation was associated to higher LDL-cholesterol levels and to higher aortic sodium homeostasis, and an α-adducin polymorphism. 

References