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GENETIC VARIATION IN *CYP4A11* AND BLOOD PRESSURE RESPONSE TO MINERALOCORTICOID RECEPTOR ANTAGONISM OR ENAC INHIBITION: AN EXPLORATORY PILOT STUDY IN AFRICAN AMERICANS

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Abstract

Background—An rs3890011 variant of *CYP4A11*, which is in linkage disequilibrium with the loss-of-function variant rs1126742, is associated with hypertension in humans. In mice, *Cyp4a* deficiency results in salt-sensitive hypertension through activation of ENaC. We tested the hypothesis that the rs3890011 variant is associated with blood pressure response to drugs acting via the ENaC pathway.

Methods and Results—African Americans with volume-dependent, resistant hypertension were randomized to treatment with placebo, spironolactone, amiloride, or combination. Blood pressure responses were analyzed by *CYP4A11* genotypes. Rs3890011 (GG:GC:CC=20:35:28) and rs1126742 (TT:TC:CC=45:31:7) were in linkage disequilibrium ($D' \approx 1$, $r = 0.561$). Expected small number of rs1126742 CC homozygotes precluded analysis of the effect of this genotype on treatment responses. Spironolactone reduced blood pressure in rs3890011 GG and GC individuals, but not in CC homozygotes ($p = 0.002$), whereas amiloride reduced blood pressure similarly in all rs3890011 genotypes. The antihypertensive effects of spironolactone and amiloride were comparable in GG and GC participants, but only amiloride reduced pressure in CC homozygotes ($-6.3 \pm 7.3 / -3.2 \pm 4.0$ versus $+6.8 \pm 7.9 / +4.8 \pm 8.6$ mmHg, $p < 0.01 / < 0.05$). The aldosterone response to spironolactone was also blunted in the CC genotype.

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Conclusions—In individuals homozygous for the *CYP4A11* rs3890011 C allele, blood pressure is resistant to mineralocorticoid receptor antagonism, but sensitive to ENaC inhibition, consistent with ENaC activation. Studies in a larger population are needed to replicate these findings.

Keywords

hypertension; epithelial sodium channel; amiloride; spironolactone

Introduction

The cytochrome P450 ω -hydroxylase *CYP4A11* oxidizes arachidonic acid to 20-hydroxyecosatetraenoic acid (20-HETE), which causes both vasoconstriction and natriuresis. (1) In humans, a variant in intron 10 of *CYP4A11* (rs3890011) with a relatively high minor allele frequency was associated with systolic blood pressure (SBP) in normotensive Chinese men ($p=0.05$ after controlling for age and body mass index), and a haplotype containing the C allele of rs3890011 was associated with hypertension in women. (2) A nearby loss-of-function variant in exon 11 of *CYP4A11* (rs1126742, T8590C, or Phe434Ser), in linkage disequilibrium with rs3890011 but with a much lower minor allele frequency (17.4%), has been associated with hypertension in several populations. (3–6) In addition, this polymorphism has been associated with the sensitivity of blood pressure to salt intake in hypertensive individuals. (7)

In mice, genetic deficiency of the ω -hydroxylase *Cyp4a10* results in salt-sensitive hypertension but does not cause a change 20-HETE synthase activity. Hypertension results from increased sodium reabsorption due to constitutive activation of the epithelial sodium channel (ENaC) mediated by a decrease in epoxygenase activity and renal synthesis of epoxyecosatrienoic acids (EETs). (8) Treatment of *Cyp4a10*^{-/-} mice with the ENaC antagonist amiloride normalizes blood pressure. This is analogous to the situation in Liddle syndrome, a Mendelian form of human hypertension in which mutations in either the β or γ subunit of ENaC result in gain-of-function and sodium retention that can be corrected by treatment with amiloride but not with spironolactone. (9,10)

The *CYP4A11* variant rs1126742 encodes for an enzyme with reduced catalytic activity, (6) making it conceivable that the associated salt-sensitive hypertension is due to deficient 20-HETE synthesis. In contrast, the mechanism for the association between the intronic variant rs3890011 and increased blood pressure is not known. We hypothesized that it may be associated with increased ENaC activity. If this were the case, the hypertension would be resistant to treatment with the mineralocorticoid receptor (MR) antagonist spironolactone but sensitive to the ENaC inhibitor amiloride. To test this hypothesis, we compared the blood pressure response to placebo, spironolactone, amiloride, and the combination of spironolactone and amiloride in an exploratory pilot study in 83 hypertensive African Americans who participated in a published randomized clinical trial, (11) and for whom DNA was available, according to the *CYP4A11* genotype at rs3890011.

Methods

The study protocol was approved by the Indiana University-Purdue University at Indianapolis institutional review board. All patients gave written informed consent.

Patients were eligible for enrollment if they were self-identified as black (defined as of African descent), between 18 and 75 years of age, and had a SBP >140 and >175 mm Hg or a diastolic blood pressure (DBP) >90 and >105 mm Hg while receiving any of the following - hydrochlorothiazide (minimum dose of 25 mg), furosemide (minimum dose of 40 mg), equivalent doses of similar diuretics and amlodipine 5 or 10 mg, or equivalent doses of a similar calcium channel blocker. (11) Any use of triamterene, an angiotensin-converting enzyme (ACE) inhibitor, or an angiotensin receptor blocker was discontinued for 1 month before the study. To restrict enrollment to patients with hypertension that was more volume dependent, patients were excluded if their plasma renin activity (PRA) exceeded 2.0 ng/mL per hour.

Protocol

Patients completed a randomized, placebo-controlled, double-blind, parallel-group trial that used a 2-by-2 factorial design, as previously described. (11) They were screened at least 3 weeks prior to randomization and eligible subjects were given 2 placebo capsules to take each morning for 3 weeks. They were then randomized to one of four treatment groups: amiloride (10 mg per day), spironolactone (25 mg per day), the combination of both drugs, and placebo. Study medications were given as two identical appearing capsules. Blood pressure measurements and blood samples were obtained at baseline and at weeks 1, 3, 5, 7, and 9. Blood pressure was measured three times in the morning prior to study medication while patients were seated; the average of the last two readings was used in the analyses.

Laboratory Analysis

Serum electrolytes and creatinine were measured using a Vitros 950 instrument (Ortho Clinical Diagnostics). PRA was measured using a radioimmunoassay for angiotensin I (Clinical Assays GammaCoat kit); the intra-assay coefficient of variation (CV) was 4.6%, and the interassay CV was 7.6%. (11) Aldosterone was also measured by radioimmunoassay (Diagnostic Products Corporation), and the intra-assay and interassay CVs were 5.4% and 13.1%, respectively.

Genetic Analysis

Genotyping for SNP rs3890011 of *CYP4A11* was performed by amplification and sequencing of a DNA segment covering exon 10 through exon 11, as previously described. (3) Genotyping for rs1126742 was also obtained to verify allele frequencies in our population.

Statistical Analysis

Data are presented as mean \pm standard deviation unless otherwise indicated. Primary end points were the changes from baseline in SBP and DBP at nine weeks of therapy. Secondary end points were changes in PRA and plasma aldosterone and in serum levels of potassium

and creatinine. Change from baseline to 9 weeks was calculated for each study participant. Deviation from Hardy-Weinberg was calculated using chi square testing. The effect of genotype on response to treatment was tested using ANCOVA, in which the age at onset of hypertension, sex, and baseline were included as covariates. Change in potassium was also included as a covariate in an ANOVA assessing the effect of rs3890011 genotype on aldosterone concentrations. Plasma aldosterone concentrations and PRA were transformed using the natural log prior to analysis. A p value less than 0.05 was considered significant.

Results

The frequencies of both rs3890011 and rs1126742 were in Hardy-Weinberg equilibrium and their genotypes were in linkage disequilibrium ($D' = 1$, $r = 0.561$, $p < 0.001$). The frequency of the minor allele for the loss-of-function variant at rs1126742 was 27.1%, similar to the previously reported 30.0% for African Americans, (3) confirming our prediction that genotypic analyses of therapeutic responses would not be possible in a population the size of ours, owing to the low number of CC homozygous subjects (TT:TC:CC=45:31:7). The only significant difference related to rs1126742 genotype was detected in all patients combined (four therapy groups). We observed a failure of aldosterone levels to increase in response to treatment in CC subjects (Figure 1), compared to TT and CT, who exhibited the expected increase in this hormone in response to MR or ENaC blockade. (12,13)

In contrast, the frequency of the minor allele for rs3890011 was 54.8%, higher in this group of African American subjects than that reported in Chinese (2) and in the 1000 Genomes database (41.5%), (14) with a genotype distribution allowing for the testing of our hypothesis (GG:GC:CC=20:35:28).

Table 1 provides baseline characteristics for subjects according to rs3890011 genotype. There was no difference in blood pressure, the number of anti-hypertensive medications, or any other baseline variable among genotypes. There was also no difference in the classes of anti-hypertensive medications used among genotype groups randomized to spironolactone or not.

Figure 2 shows that placebo-induced changes in blood pressure over the period of study were not different among the three rs3890011 genotypes (panel A). There was no reduction in SBP and DBP in response to spironolactone monotherapy in the 5 CC homozygous subjects, significantly different from the reductions observed in the 7 GG and 8 GC (panel B). In contrast, amiloride monotherapy reduced SBP and DBP similarly in all *CYP4A11* rs3890011 genotypes (GG:GC:CC=6:8:6, panel C). In a sensitivity analysis, there was a significant association between *CYP4A11* rs3890011 genotype (GG:GC:CC=12:15:13) and blood pressure response to any spironolactone-containing treatment (Figure 3) but not to any amiloride-containing therapy ($p > 0.15$, not shown). Finally, in all subjects carrying at least one rs3890011 G allele (GG+GC) amiloride reduced SBP and DBP ($-10.6 \pm 8.2 / -5.9 \pm 6.4$ mmHg) to the same extent as spironolactone ($-9.8 \pm 9.4 / -6.3 \pm 6.5$ mmHg), $p = 0.41 / 0.43$. In contrast, in CC homozygous subjects amiloride reduced SBP and DBP ($-6.3 \pm 7.3 / -3.2 \pm 4.0$ mmHg) to a greater extent than spironolactone did ($+6.8 \pm 7.9 / +4.8 \pm 8.6$), $p < 0.01 / < 0.05$. The increase in SBP of CC with spironolactone monotherapy was significant ($p < 0.03$) but not

significantly different from that sustained by all subjects given placebo during the trial (3.6 ± 11.3), $p=0.28$. There was no effect of gender or BMI on response to spironolactone or amiloride, alone or in combination.

Aldosterone responses to therapy followed a similar pattern. In subjects carrying at least one G allele (GG+GC), amiloride increased aldosterone from 452 ± 58 to 784 ± 97 pmol/L and spironolactone increased aldosterone from 352 ± 53 to 596 ± 91 pmol/L (drug comparison not significant, adjusted marginal means after controlling for gender and change in potassium). In contrast, in CC homozygous subjects, amiloride increased aldosterone from 585 ± 89 to 989 ± 144 pmol/L, whereas spironolactone did not affect aldosterone (from 446 ± 91 to 449 ± 147 pmol/L, $p=0.038$ for drug comparison). There were no other significant relationships between *CYP4A11* rs3890011 and the effect of either spironolactone or amiloride on heart rate, PRA, plasma sodium, plasma potassium, or creatinine.

Discussion

Gain-of-function mutations in ENaC such as occur in Liddle syndrome result in salt-sensitive hypertension that is resistant to MR antagonists but responds to the ENaC inhibitor amiloride. (9,10) In rodent models, decreased expression of *Cyp4a* also results in increased ENaC activity and salt-sensitive hypertension that is responsive to amiloride. (8,15) We report for the first time in this exploratory pilot study that African Americans with resistant, volume-dependent hypertension who are homozygous for the C allele at rs3890011 of *CYP4A11*, an allele previously associated with blood pressure (2), are resistant to the blood pressure lowering effects of spironolactone but respond to amiloride.

Several lines of evidence in rodents suggest that CYP4A modulates ENaC function. First, Nakagawa et al reported that genetic deficiency of *CYP4a10* results in constitutive activity of ENaC and salt-sensitive hypertension that is responsive to amiloride. (8) This is thought to result from decreased formation of EETs that act as endogenous inhibitors of ENaC. In the Dahl salt-sensitive rat, *CYP4A* genotype segregates with salt-sensitive hypertension. (16) Decreased CYP4A hydroxylase activity results in salt-sensitive hypertension in this model, whereas induction of CYP4A increases pressure natriuresis. More recently, Kakizoe et al have reported that β and γ ENaC subunit mRNA and protein are overexpressed in Dahl salt-sensitive rats. (15) They further reported that 4-week treatment with amiloride reduced blood pressure and renal injury whereas treatment with eplerenone did not.

A common intronic variant of *CYP4A11*, rs3890011, is associated with blood pressure in the Chinese. (2) We studied the association between this variant and the blood pressure responses to amiloride and spironolactone in our population. The rs3890011 locus is 247 base pairs away and in linkage disequilibrium with rs1126742, a rarer coding variant of *CYP4A11*. The latter variant in exon 11 encodes for a thymidine-to-cytosine substitution at base pair 8590 that results in a Phe-to-Ser substitution at amino acid 434 and loss of function of the product enzyme. (3) The low frequency of the minor allele of rs1126742 precludes genotypic analyses in populations the size of ours, owing to the small number of homozygous CC subjects. Therefore, we did not study the relationship between this genotype and blood pressure responses to therapy in our small clinical trial. We were only

able to document that the aldosterone response to treatment was diminished in rs1126742 CC homozygous subjects.

Whether there is a functional consequence of the rs3890011 intronic variant is not currently known but the much larger frequency of its minor allele allowed us to examine the relationship between this *CYP4A11* polymorphism and the blood pressure response to spironolactone and amiloride in African Americans with resistant hypertension and suppressed PRA. The prevalence of salt-sensitive or volume-dependent hypertension is increased among African Americans. (17) Genetic variants in ENaC that have been associated with an increased urinary aldosterone/potassium ratio, an index of ENaC activity, are more common in African Americans but have not been universally associated with hypertension. (18,19) As previously published in this study population of African Americans with volume-dependent resistant hypertension, amiloride alone has a greater effect on blood pressure than does spironolactone alone. (11) Whereas it is possible that this relates to the doses of spironolactone and amiloride used, our data suggest that this is also attributable to a diminished response to spironolactone in subjects homozygous for the *CYP4A11* rs3890011 C allele.

The loss-of-function *CYP4A11* rs1126742 C allele is more common in African Americans than in other ancestral groups (3) and we found the *CYP4A11* rs3890011 C allele to be more common in our African American study population than previously reported in Chinese. (2) Whether the findings of this study will hold up in individuals with resistant hypertension from other racial groups remains to be studied.

A limitation of our study is the small number of subjects, although the sensitivity analysis in which we combined subjects in spironolactone treatment groups (Figure 3) supports the conclusions. Also, baseline PRA and aldosterone concentrations were not significantly lower in rs3890011 CC homozygotes, as one would expect if ENaC activity were increased. The exclusion of subjects with a PRA greater than 2.0 ng/mL per hour and the use of concomitant medications that affect PRA (e.g., diuretics and β -blockers) may have confounded any relationship between *CYP4A11* genotype and baseline PRA. Williams et al previously reported the relationship between the *CYP4A11* rs1126742 genotype and salt-sensitivity of blood pressure in untreated subjects. (7) In that study, although there was no significant relationship between genotype and PRA or aldosterone overall, there was a significant relationship between *CYP4A11* genotype and aldosterone in African Americans during low salt (16.5 ± 9.5 , 14.9 ± 7.2 , and 7.9 ± 3.4 ng/dL, in 36 TT, 33 CT, 5 CC, respectively, $p=0.03$, unpublished). There was no relationship between *CYP4A11* genotype and PRA in African Americans in that study (unpublished).

In summary, inappropriate activation of ENaC has increasingly been recognized as a feature of resistant hypertension. (20) The MR antagonists spironolactone and eplerenone are often effective in reducing blood pressure in patients with resistant hypertension. (21,22) Not all patients respond to MR antagonism, however, and in these patients amiloride may be more effective. (11) Genetic variants in *CYP4A11* have been associated with hypertension and with salt-sensitive hypertension in particular. (2–7) The present data suggest that genetic

variation in *CYP4A11* might be used to predict individuals who should be treated with a direct ENaC inhibitor. Additional studies are needed to replicate these findings.

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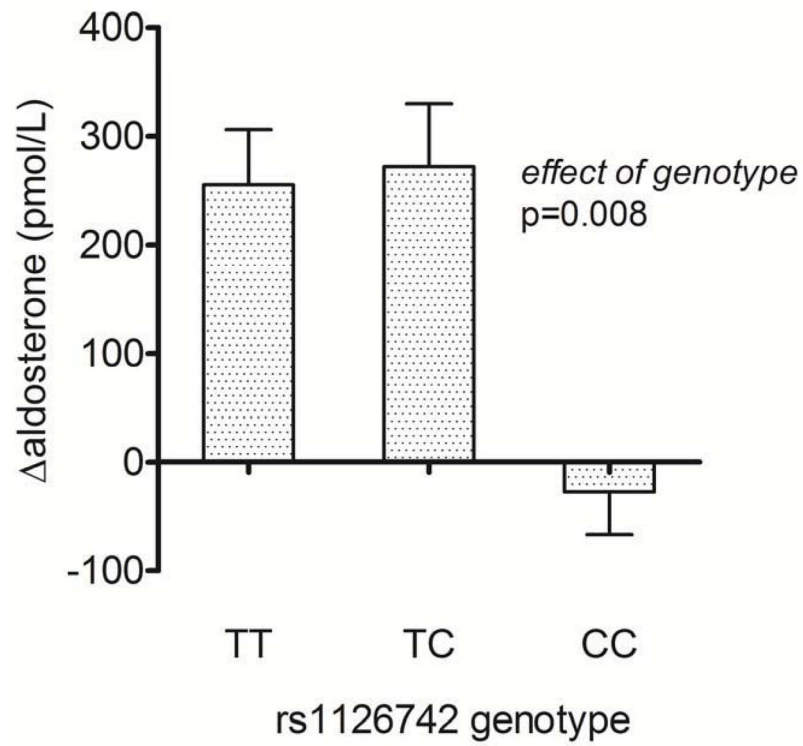


Figure 1. Relationship between *CYP4A11* rs1126742 (T8590C) genotype and aldosterone response to any therapy including placebo, spironolactone, amiloride or combination amiloride and spironolactone. Data are presented as means \pm standard error.

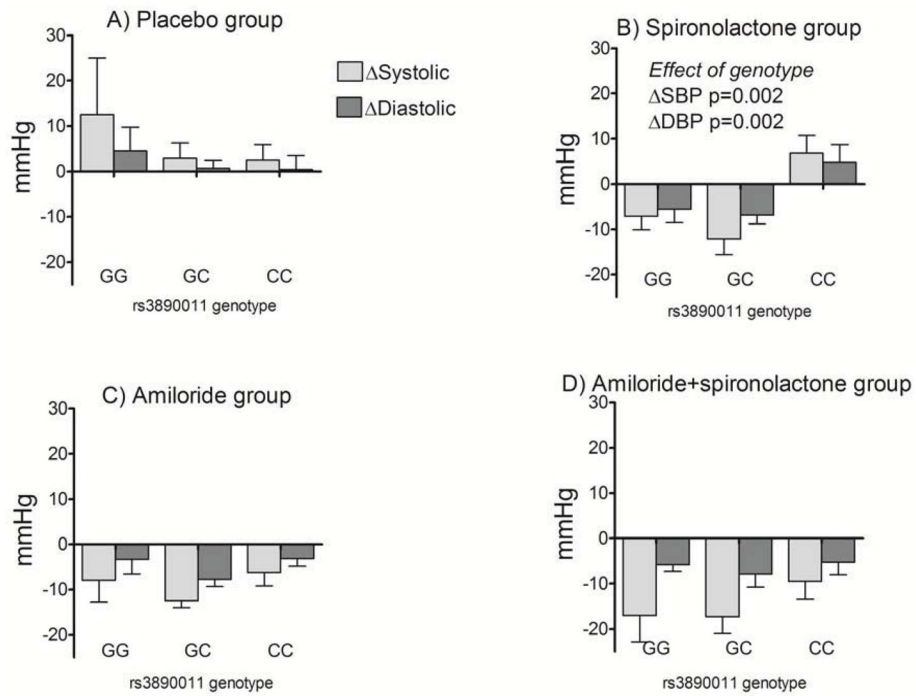


Figure 2. Relationship between *CYP4A11* rs3890011 genotype and blood pressure response after nine weeks of treatment with (A) placebo, (B) spironolactone, (C) amiloride or (D) combination amiloride and spironolactone. Data are presented as means \pm standard error.

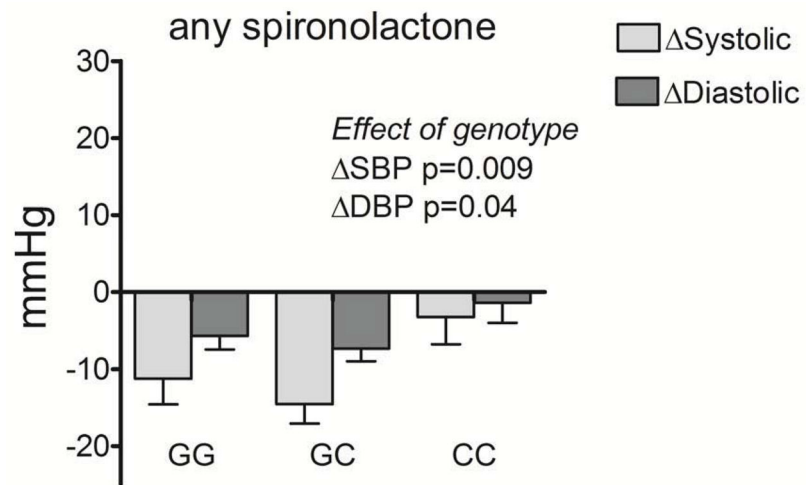


Figure 3. Relationship between *CYP4A11* rs3890011 genotype and blood pressure response to spironolactone in the presence or absence of amiloride (any spironolactone). Data are presented as means \pm standard error.

Table 1Baseline subject characteristics by *CYP4A11* rs3890011 genotype

<i>CYP4A11</i> rs3890011 Genotype	GG (N=20)	GC (N=35)	CC (N=28)
Gender, M:F	12:8	16:19	16:12
Age, years	49.0±9.0	44.5±9.9	47.9±8.7
Body Mass Index, kg/M²	32.8±4.9	34.3±7.4	35.4±10.0
Duration of Hypertension, years	13.2±8.2	8.8±7.9	12.2±8.1
Number of Anti-Hypertensives	2.35±0.67	2.29±0.46	2.21±0.50
Class of Anti-Hypertensives, N (%)			
Thiazide diuretic	19 (95.0%)	31 (88.6%)	25 (89.3%)
Loop diuretic	1 (5.0%)	4 (11.4%)	3 (10.7%)
Amlodipine or other dihydropyridine	19 (95.0%)	33 (94.3%)	28 (100.0%)
CCB other	1 (5.0%)	1 (2.9%)	0
β-blocker	6 (30.0%)	10 (28.6%)	6 (21.4%)
Alpha-2 agonist	2 (10%)	0	0
Minoxidil	0	0	1 (3.8%)
Alpha-1 antagonist	0	1 (2.8%)	1 (3.8%)
Systolic Blood Pressure (mmHg)	145.8±12.1	139.9±10.5	140.9±12.8
Diastolic Blood Pressure (mmHg)	91.5±7.8	92.1±6.0	89.4±8.9
Heart Rate (bpm)	79.4±9.2	75.5±10.3	78.5±8.8
Glucose (mg/dL)	123.6±39.0	105.7±30.8	117.4±46.4
Serum sodium (mmol/L)	141.8±2.0	142.1±1.9	141.6±2.4
Serum potassium (mmol/L)	3.6±0.4	3.6±0.4	3.7±0.3
Serum creatinine (mmol/L)	90.2±15.0	91.9±18.6	92.8±24.8
Plasma Renin Activity (ng/mL/min)	0.89±1.90	0.77±1.87	0.64±0.96
Aldosterone (pmol/L)	401.7±232.7	407.2±193.9	423.8±205.0