Combination of Renin-Angiotensin System Polymorphisms Is Associated With Altered Renal Sodium Handling and Hypertension

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Abstract—Genes of the renin-angiotensin–aldosterone system (RAAS) are natural candidates for sodium homeostasis and blood pressure regulation. To investigate the effect of a combination of polymorphisms of RAAS genes on renal sodium handling and blood pressure, 918 participants to the Olivetti Heart Study were genotyped for the following polymorphisms: I/D of angiotensin converting enzyme (*ACE*), M235T of angiotensinogen (*AGT*), A1166C of angiotensin II type-1 receptor (*AT1R*), and C-344T of aldosterone synthase (*CYP11B2*). The segmental renal sodium handling was evaluated by the fractional excretions of exogenous lithium (FE-Li), uric acid (FE-UA), and sodium (FE-Na). Twenty-eight carriers of triple homozygosity for M (*AGT*), A (*AT1R*), and C (*CYP11B2*) in the presence of the D allele of *ACE* (DD/ID) showed lower FE-Li (20.0%±5.9% versus 25.0%±7.5%; $P=0.004$; mean±SD), FE-UA (6.3%±2.0% versus 8.2%±2.7%; $P=0.001$), and FE-Na (0.96%±0.41% versus 1.22%±0.61%; $P=0.004$) as compared with all other allelic combinations (n=890), independently from age and body mass, suggesting an enhanced rate of proximal tubular sodium reabsorption. The carriers of the MM, AA, CC, DD/ID combination showed a substantially higher probability of being hypertensive (OR: 3.4 [(99% CI: 1.1 to 10.1]), independently of age and body mass. This relatively rare combination of allelic variants of candidate genes of the RAAS is associated with a significant alteration in proximal renal sodium handling and with higher risk of hypertension, suggesting that a combination of polymorphic variants at different candidate loci may affect phenotypic expression even in the absence of detectable effects of each variant at any single locus. **(*Hypertension*. 2004;43:598-602.)**

Key Words: renal circulation ■ blood pressure ■ hypertension ■ genes ■ polymorphism ■ renin-angiotensin–aldosterone system

According to Guyton's hypothesis,[1] modifications of the pressure–natriuresis relation at the kidney level are causally involved in the development and maintenance of high blood pressure, regardless of the initial pathogenic factor. Approximately 20 years ago, de Wardener and MacGregor suggested that an inherited alteration of the physiological capacity of the kidney to excrete sodium or to adapt to changes in sodium intake participates in the pathogenesis of some forms of hypertension.[2] Despite intensive investigation, the renal defects responsible for impaired sodium excretion have not been fully elucidated. Among the important determinants of renal sodium handling, eg, renal hemodynamic, humoral, and neural factors, it is conceivable that genetic factors might explain, at least in part, the alterations observed.

The definition of the genetic basis of common forms of the so-called essential hypertension must be inevitably accompanied by the search for physiological mechanisms that underlie blood pressure regulation. Alterations of genes involved in the fluid and sodium homeostasis play a causative role in rare forms of monogenic hypertension, producing an increase in tubular sodium and water reabsorption and, finally, a chronic blood pressure increase.[3] Although polymorphisms in genes that represent the natural candidates for alteration of sodium homeostasis, such those of the renin-angiotensin–aldosterone system (RAAS), have been extensively tested as genetic determinants of arterial hypertension with conflicting results,[4–7] only rarely has their influence on intermediate phenotypes of renal sodium handling been addressed. The difficulty to draw clear conclusions regarding the impact of

Received May 7, 2003; first decision, May 29 2003; revision accepted November 26, 2003.

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Hypertension **is available at http://www.hypertensionaha.org** **DOI: 10.1161/01.HYP.0000117985.57001.b3**

TABLE 1. Genotype and Allele Frequencies for Angiotensin-Converting Enzyme (*ACE*), Angiotensinogen (*AGT*), Angiotensin II Type 1 Receptor (*AT1R*) and *CYP11B2*

Gene (Polymorphic Locus)	Genotype N (%)			Allele Frequencies	
ACE (Intron 16 I/D)	DD 371 (40.3)	ID 411 (44.7)	II 136 (14.8)	D 0.63	I 0.37
AGT (M235T)	MM 278 (30.3)	MT 444 (48.4)	TT 196 (21.4)	M 0.55	T 0.45
AT1R (A1166C)	AA 495 (53.9)	AC 360 (39.2)	CC 63 (6.9)	A 0.74	C 0.26
CYP11B2 (T-344C)	TT 243 (26.5)	TC 465 (50.7)	CC 210 (22.9)	T 0.52	C 0.48

Values are number percent.

specific genetic variants on blood pressure regulation and sodium homeostasis is probably because of poorly understood gene–gene[8] and/or gene–environment interactions, poorly defined phenotypes, and the lack of studies based on reliable models for the measurement of segmental renal sodium handling in population settings.

The study of segmental tubular sodium handling by measurement of the clearance of exogenous or endogenous lithium has been a source of valuable information regarding the effects of genetic and metabolic alterations of renal tubular function and blood pressure regulation in humans.[9,10] The clearance of lithium is the best available indicator of proximal tubular sodium handling in vivo. With this technique, an increased proximal reabsorption of sodium has been demonstrated in animal model of hypertension,[11] in salt-sensitive individuals,[12,13] and in centrally obese hypertensive patients.[14] Only a few studies have investigated the effects of polymorphic variants of candidate hypertension genes on the segmental tubular sodium handling. In particular, with the use of the lithium clearance technique, we[15] and others[16] have recently shown that polymorphic variants of candidate hypertension genes are associated with increased proximal sodium reabsorption.

The goal of the present analysis was to assess the effect of a combination of variants at different loci of genes, all lying along a specific physiopathological pathway (the RAAS) on proximal tubular sodium reabsorption and blood pressure regulation in a sample of Italian men.

Methods

The selection of the population has been described elsewhere.[15,17] An extended Methods section can be found in an online supplement available at http://www.hypertensionaha.org. In brief, 1075 men (age 25 to 75 years) were examined between May 1994 and December 1995. The study had ethical approval and participants consented to participate. Anthropometry, sitting blood pressure (by random zero sphygmomanometer), blood and urine tests, and a questionnaire including adherence to a low-salt diet and current pharmacological treatment for hypertension were obtained as previously described.[15,17] Hypertension was defined as a pressure ≥140 mm Hg systolic and/or ≥90 mm Hg diastolic, matching the 1999 WHO–ISH definition.[18] The clearance study was performed according to procedures extensively validated in our laboratory.[9,17,19,20] Standard formulae were used to calculate the clearance of creatinine, sodium, lithium, and uric acid, and results were expressed as fractional excretions (%).[9,17,19,20] The fractional excretion of exogenous lithium was measured in 677 subjects who did not differ from the remaining participants. Plasma aldosterone was measured by radioimmunoassay (DRG Instruments, GmbH-Germany) in 661 participants, for whom a plasma sample was available, regarding their habitual diet. Genotyping of four RAAS polymorphisms was performed in 918 participants. Polymorphisms I/D of the *ACE*, T235C of the angiotensinogen gene, A1166C of the angiotensin II type 1 receptor gene, and C-344T in the 5′ flanking region of *CYP11B2* were typed as previously described.[21–25] For each polymorphism, a 10% random sample of the study population was genotyped twice in a blinded fashion with concordant results. Genetic data were analyzed for Hardy-Weinberg equilibrium and for allelic frequency using the Tools for Population Genetic Analysis version 1.3 (available at http://bioweb.usu.edu/mpmbio/index.htm). Statistical analysis (SPSS 11.0; Chicago, Ill) followed a 2-step approach. First, the influence of each candidate gene variant at any single locus on proximal tubular sodium handling and blood pressure was assessed by analysis of covariance. Second, to assess the effect of simultaneous variation in different RAAS genes, we considered all possible combinations of the 4 variants, thus obtaining 81 genotypes. Of them, 5 had empty cells and 28 had fewer than 6 individuals in a cell, thus making a comparison of all genotype combinations impracticable, despite the relatively large sample size. We then built a model based on two assumptions. First, we focused essentially on the homozygous condition by assuming that the effect, if any, of multiple variants of candidate genes would be more evident in the presence of a double copy of the allele. Second, we selected either the alleles whose association with phenotypes of interest (blood pressure and/or electrolyte metabolism) has been shown in previous analyses on this population (the C allele of the *CYP11B2*[25] and the D allele of the *ACE*[26]) or the more frequent alleles in this population (the M allele of *AGT* and the A allele of *AT1R*) (Table 1) to obtain groups large enough to allow for sufficient statistical power. Because only 9 subjects carried this complete homozygosity combination, further analyses were performed by adding the heterozygotes at each single locus in separate models. We obtained four possible combinations that were, in turn, compared with the remaining population. To reduce the false-positive results, more stringent criteria were adopted for the statistical evaluation. Because four different comparisons were made, type 1 error was set at $0.05 \div 4 = 0.0125$, and 99% CI were used. Fisher exact test was used to assess the independence in 2×2 tables when cells had an expected frequency <5. The statistical significance of between-group differences for continuous variables was assessed by two-sided unpaired *t* test. Multiple linear regression models were calculated to assess whether the allelic combination obtained made a statistically significant contribution to the variability of renal tubular sodium handling, allowing for confounders. Logistic regression was used to determine the predictive value of allelic combinations with regard to the occurrence of hypertension, accounting for confounders. Results were expressed as mean and SD or 99% CI, as specified.

Results

Included in the analysis were 918 participants (age 51.7 ± 7.3 years; body mass index 26.9 ± 3.0 kg/m^2; systolic blood pressure 130.1 ± 17.4 mm Hg; diastolic blood pressure 84.1 ± 9.9 mm Hg). The prevalence of hypertension (defined previously) was 41.3%. Table 1 shows genotype and allele frequency for each variant. All four polymorphisms were in Hardy-Weinberg equilibrium. No significant association was observed between RAAS variants at any single locus and indexes of renal sodium handling.

The next step of the analytical procedure examined the effect of concomitant homozygosity for the D, M, A, and C

TABLE 2. Indices of Tubular Sodium Handling: Comparison Between Carriers of MM AA CC DD/ID and All Other Allelic Combinations

Indices of Tubular Sodium Handling	MM AA CC DD/ID	All	*P* Value	Difference (99% CI)
Lithium FE (%)*	20.0±5.9	25.0±7.5	0.004	−4.9 (−9.3 to −0.6)
Uric Acid FE (%)	6.3±2.0	8.2±2.7	0.001	−1.9 (−3.3 to −0.4)
Sodium FE (%)	0.96±0.41	1.22±0.61	0.004	−0.26 (−0.5 to −0.02)

Values are Mean±SD.

MM AA CC DD/ID indicates homozygosity for M allele of angiotensinogen (*AGT*), A allele of angiotensin II type 1 receptor (*AT1R*), and C allele of *CYP11B2* in presence of D allele of *ACE*; FE, fractional excretion.

*Data available for 677 individuals.

variants of the 4 genes. This condition was associated with a 2-fold higher frequency of hypertension as compared with the remaining population (80% versus 40%; $P=0.05$) and with markedly lower FE of lithium (19.7%±6.2% versus 24.9%±7.5%), uric acid (6.9%±1.7% versus 8.2%±2.7%), and sodium (1.0%±0.3% versus 1.2±0.6%). Then, we analyzed the effect of the possible combinations, obtained by alternatively adding to the 9 full homozygous subjects the participants heterozygous at any single locus.

Of the 4 resulting genotypic combinations (MM/MT, AA, CC, DD; MM, AA/AC, CC, DD; MM, AA, CC/CT, DD; and MM, AA, CC, DD/ID), only the condition of triple homozygosity for M (*AGT*), A (*AT1R*), C (*CYP11B2*) in the presence of the D allele of the *ACE* gene (DD/ID), observed in 28 individuals (3% of the sample), was associated with significantly lower fractional excretion of lithium and uric acid in comparison with the remaining population (Table 2), thus suggesting an enhanced rate of proximal sodium reabsorption in these individuals. They also showed a significantly lower fractional excretion of sodium (Table 2) and a trend to higher plasma aldosterone concentration (360±262 pmol/L versus 274±151 pmol/L; $P=0.05$), but no difference in age (51.6±6.3 versus 51.7±7.3 years), body mass index (27.4±3.1 versus 26.9±3.0 kg/m^2), dietary sodium intake (estimated from food frequency questionnaires), and proportion of individuals reporting adherence to a reduced-salt diet. Pharmacological treatment for hypertension was more frequent in the 28 carriers of the MM, AA, CC, DD/ID combination (32.1% versus 16.6%; Fisher exact test=0.04). No difference in the use of any major class of antihypertensive drug was found between the 2 groups; in particular, none of the carriers of the MM, AA, CC, DD/ID combination reported the use of diuretics, which might be associated with more avid renal tubular sodium reabsorption. However, the exclusion from the analysis of all the subjects undergoing diuretic treatment did not affect the results.

The relationship of the indexes of renal tubular sodium handling to the MM, AA, CC, DD/ID genotype was further explored by multiple regression analysis, adjusting for the concomitant effect of age, body mass index, low-salt diet, and ongoing pharmacological treatment. As shown in Table 3, the MM, AA, CC, DD/ID genotype was a significant predictor of the variability in the fractional excretion of lithium and uric acid, independently of confounders.

Finally, we analyzed the effect of the MM, AA, CC, DD/ID combination on blood pressure levels and on the prevalence of hypertension. The association with blood pressure was evaluated after the exclusion of participants undergoing pharmacological treatment for hypertension (n=161). The 19 carriers of the MM, AA, CC, DD/ID not using pharmacological treatment had significantly higher age- and body mass index-adjusted blood pressure as compared with the 738 untreated participants with different genotypes (systolic: 136.8±18.2 versus 126.9±15.5 mm Hg, $P=0.006$; diastolic: 87.6±9.5 versus 82.5±9.1, $P=0.01$). Furthermore, the MM, AA, CC, DD/ID genotype was associated with a higher probability of hypertension (blood pressure >140/90 mm Hg or current pharmacological treatment for hypertension, [67.9% versus 40.4%; OR: 3.4; 99% CI: 1.1 to 10.1]), independent of age and body mass. The population-attributable risk of hypertension, ie, the proportion of hypertension attributable to the presence of this genetic combination, is 6.8%.

TABLE 3. Multivariate Regression Equations, Fractional Excretion of Lithium and Uric Acid as Dependent Variables

	Lithium FE		Uric Acid FE	
Explanatory Factor	B (99% CI)	*P* Value	B (99% CI)	*P* Value
Allelic combination*	4.37 (0.12 to 8.6)	0.008	1.88 (0.41 to 3.34)	0.001
Age (y)	−0.08 (−0.18 to 0.02)	0.054	0.003 (−0.03 to 0.03)	0.832
BMI (kg/m^2)	−0.33 (−0.57 to −0.08)	0.001	−0.17 (−0.25 to −0.09)	<0.001
Low-salt diet (yes no)	1.13 (−1.13 to 3.39)	0.197	0.44 (−0.30 to 1.18)	0.125
Drug treatment (yes no)	−2.77 (−4.99 to −0.54)	0.001	−0.57 (−1.29 to 0.14)	0.04

FE indicates fractional excretion; BMI, body mass index.

*Homozygosity for M allele of angiotensinogen (*AGT*), A allele of angiotensin II type 1 receptor (*AT1R*), and C allele of *CYP11B2* in presence of D allele of *ACE* vs all other allelic combinations.

Discussion

In this population-based study, we showed that the relatively rare condition of triple homozygosity for M (*AGT*), A (*AT1R*), and C (*CYP11B2*) in the presence of the D allele of *ACE* is associated with a greater prevalence of hypertension and with an abnormality in renal sodium handling evaluated by the fractional excretion of exogenous lithium and of uric acid.

In the carriers of the MM, AA, CC, DD/ID genotype, fractional excretions of lithium and uric acid were significantly lower than in all other allelic combinations irrespective of age, body mass, ongoing pharmacological treatment, and adherence to a low-salt diet. These data indicate a trend to an enhanced rate of sodium reabsorption at a tubular proximal site. In the experimental conditions of our test, the fractional excretion of sodium, reflecting sodium handling through the entire nephron, was also reduced in the carriers of this allelic combination, suggesting that the more avid sodium reabsorption occurring at the proximal tubular level was not fully compensated for by enhanced sodium rejection at more distal sites. Indeed, the higher levels of plasma aldosterone found in the 28 individuals carrying this particular genotype are in keeping with this finding. It can be speculated that the lower sodium load reaching the macula densa as a consequence of the enhanced rate of proximal reabsorption modulates the tubulo-glomerular feedback mechanism in a way that favors an increased glomerular perfusion and a stimulation of renin secretion and, in turn, of aldosterone production. It is to be expected, however, that over the 24 hours, a neutral sodium balance should be achieved possibly through the effect of higher blood pressure and an increase in the sodium filtered load.

Although statistical associations do not prove cause–effect relationships, the higher rate of sodium reabsorption at the proximal tubule observed in the carriers of the MM, AA, CC, DD/ID combination provides a plausible mechanism to explain the greater prevalence of hypertension in these subjects. An alteration in renal tubular sodium handling is the common pathway leading to higher (more rarely lower) blood pressure in "monogenic" disorders such as glucocorticoid-remediable aldosteronism, Liddle syndrome, apparent mineralocorticoid excess syndrome, and Gitelman and Bartter syndromes.[3] Increased susceptibility to high blood pressure has been reported in individuals carrying common variants of hypertension-candidate genes affecting renal tubular sodium handling, eg, the Gly460Trp variant of the gene encoding for the α-adducin cytoskeleton protein,[16] the beta subunit of the sodium channel in blacks,[27,28] and the more rare Gly40Ser variant of the glucagon receptor gene.[15]

The RAAS influences renal sodium handling and vascular volume homeostasis,[29] and polymorphic variants in the genes encoding for the components of the RAAS are considered prime candidates for inducing susceptibility to high blood pressure.[30] The studies on the effects of single genetic markers of the RAAS on the "blood pressure" or on the "hypertension" phenotype, however, have led to conflicting results.[4–7] An explanation for these inconsistencies can be found in the occurrence of gene–gene interactions that may obscure the detection of the contribution of a single marker with a modest effect on a complex phenotype. Thus, the phenotypic expression of essential hypertension may be attributed to the interaction between a large number of "susceptibility genes" of variable penetrance and a triggering environmental stimulus, such as high-salt intake.[31]

Limitations of the Study

The present study has limitations, some of which are inherent to the use of genetic association for the study of multifactorial common diseases,[32] and some others are related to the methodological constraints posed by a large-scale epidemiological investigation such as the Olivetti Heart Study. Although the lithium clearance and, to a lesser extent, that of uric acid are the best available indicators of segmental tubular sodium handling in vivo,[33–35] they provide only an indirect estimate of the rate of proximal tubular sodium transport. The protocol adopted in the present study was nevertheless validated under various experimental conditions by our group.[9,17,19,20] Expressing the renal clearance of lithium and uric acid as fractional excretion provides a measure that is independent of the glomerular filtration rate and of possible sources of bias, such as differences in flow rate and incomplete urine collection.[19] Because the Olivetti cohort comprises only male white participants, our conclusions cannot be generalized to populations other than white Italian men. Because high blood pressure was largely prevalent in the majority of the Olivetti participants bearing different genotypes, the allelic combination described can explain only a small proportion (estimated as 6.8%) of the hypertension occurring in our study population. The statistical analysis was based on a "hypothesis-generating" approach that was justified by the need to overcome the bottleneck of a low number of carriers of 4 homozygous variants of genes, all involved in the same physiopathological pathway. However, the adjustment introduced in the level of probability required to assess statistical significance and the internal consistency of results across several outcomes reduce the likelihood that results were caused by chance alone. The measurement of plasma renin activity (not performed at the time) and a more rigorous standardization of plasma aldosterone measurement might have been useful to gain further insight into the possible mechanisms of the phenomena observed.

As recently reported by Lohmueller et al,[32] the results of the present study, and of other studies reporting positive associations between genetic variants and phenotypes of complex diseases, may be overestimated and/or caused by a false-positive association. However, because of the inadequacy of statistical methods available to explore the concomitant effects (if any) of more genetic variants on a given phenotype, the possibility exists that true biological associations involving the interaction of multiple variants at different loci also may be underestimated in the presence of a large well-characterized study population. Hopefully, the replication of these results in other population samples will rule out the possibility that they are restricted to the population examined or dependent on methodological and sampling flaws.

Conclusions

In conclusion, we have reported that in an Italian male adult population, triple homozygosity for M (*AGT*), A (*AT1R*), and C (*CYP11B2*) in the presence of the D allele of *ACE* was

associated with a significantly higher proximal tubular sodium reabsorption and with a greater prevalence of hypertension, suggesting that this allelic combination may be considered a renal "hypertension-leading haplotype," likely to play a role in the presence of high-salt intake. These findings confirm that the in vivo investigation of segmental tubular sodium handling may provide a valuable contribution to the identification of the pathways and mechanisms involved in the long-term regulation of blood pressure, and they support the view that genetic or acquired alterations of renal sodium handling may be an important cause of blood pressure variability in humans.

Perspectives

Although the identification of loci (or combination of loci) having small effects on blood pressure variability in the population may be of little help to the diagnosis and treatment of hypertension in individual patients, the construction of models of increased complexity for the study of candidate genes may be an important step toward the identification of pathways and molecular mechanisms involved in the development of multi-factorial traits such as hypertension.

Acknowledgments

The study was supported in part by grants from MURST (Italian Ministry of University and of Scientific and Technological Research COFIN 1998 and 2000) and by Modinform s.p.a. (Olivetti group). The authors thank the Olivetti employees for their enthusiastic participation. The authors also thank the reviewers for their many constructive comments on the earlier versions of the manuscript. F.P.C. is a member of the St. George's Cardiovascular Research Group. A.V. is the recipient of a research grant from the IMMIDIET project (EC Fifth Framework Program, contract QLK1-CT-2000).

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