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Abstract—We analyzed the association between salt sensitivity in essential hypertension and 8 genetic polymorphisms in 6 genes of the renin-angiotensin aldosterone system. Seventy-one patients with essential hypertension were classified as salt sensitive or salt resistant by means of the 24-hour ambulatory blood pressure (BP) change to high salt intake. The polymorphisms evaluated correspond to the following genes: ACE (I/D), angiotensinogen (M235T), angiotensin II type 1 receptor (A1166C), 11β-Hydroxysteroid dehydrogenase type 2 (11βHSD2) (G534A), aldosterone synthase (C-344T and Intron 2 conversion), and the mineralocorticoid receptor (G3514C and A4582C); all were determined using standard polymerase chain reaction methods. Thirty-five patients (49%) were classified as salt sensitive. We analyzed the BP response to high salt intake among genotypes and found a significant association for ACE I/D and 11βHSD2 G534A polymorphisms. Patients homozygous for the insertion allele of the ACE gene (II) had a significantly higher BP increase with high salt intake than did patients homozygous for the deletion allele (DD). Heterozygous patients (ID) exhibited an intermediate response. The prevalence of salt-sensitive hypertension was also significantly higher ($P=0.003$) in II (68%) and DI patients (59%) compared with DD hypertensives (19%). With respect to 11βHSD2 G534A, patients with the GG genotype had a significantly higher systolic BP increase with high salt intake than did GA patients. In addition, plasma renin activity suppression in response to high salt was significantly greater in GA patients than in GG patients. The prevalence of salt-sensitive hypertension was 14.3% in GA patients and 50.8% in GG patients ($P=0.067$). In conclusion, the I allele of ACE I/D polymorphism is significantly associated to salt-sensitive hypertension. The BP response to high salt intake was different among genotypes of ACE I/D and 11βHSD G534A, suggesting that these polymorphisms may be potentially useful genetic markers of salt sensitivity. **(*Hypertension*. 2001;38:1204-1209.)**

Key Words: angiotensin-converting enzyme ■ angiotensinogen ■ receptors, angiotensin ■ mineralocorticoids ■ aldosterone ■ genes ■ polymorphism ■ sodium

Essential hypertension is a complex syndrome determined by both genetic and environmental factors. In the past several years, a number of candidate genes have been tested for association with this disease, with controversial results. More recently, the importance of studying the gene-gene and gene-environment interactions has been highlighted. In this sense, the blood pressure (BP) response to increased dietary salt constitutes a capital phenotype in the study of the gene-environment interactions in essential hypertension. On the one hand, this phenotype is heterogeneous among individuals, and thus, ≈50% of patients with hypertension are found to be salt sensitive. On the other hand, there is evidence that salt sensitivity may be genetically determined, because normotensive and hypertensive salt-sensitive subjects tend to exhibit familial history of hypertension more frequently than do those who are salt resistant,[1] and in addition, there is a familial resemblance in the BP response to sodium restriction.[1,2]

Although many factors influence the BP response to high-salt diet, the renin-angiotensin-aldosterone system (RAAS) has been demonstrated to play a central role.[3] Low-renin hypertensives show an increased BP response to NaCl load,[4] and salt-sensitive individuals exhibit a blunted response of the RAAS when switching from low to high salt intakes, compared with that of salt-resistant subjects.[5] Polymorphisms in candidate genes of this system have been extensively tested as genetic determinants of essential hypertension in the past several years. Some of the polymorphisms that have been associated with hypertension are the ACE I/D (D allele, only in males),[6] the angiotensinogen M235T (T allele),[7] and the aldosterone synthase C-344T (T allele).[8] In addition, these polymorphisms determine part of the variations in plasma levels of ACE, angiotensinogen, and aldosterone, respectively.[9–11]

The mineralocorticoid receptor gene (MLR) and the 11β-hydroxysteroid dehydrogenase type 2 gene (11βHSD2) have also been considered as candidate genes for essential hypertension because they play a causative role in rare forms of monogenic hypertension or hypotension.[12] Mutations in the

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former gene cause pseudohypoaldosteronism type I, a monogenic syndrome of hypotension and salt wasting and a rare form of hypertension, whereas mutations in the latter cause the apparent mineralocorticoid excess, a form of salt-sensitive monogenic hypertension.[12]

Essential hypertension, as well as the target organ damage and other associated phenotypes, such as salt sensitivity, appear to be polygenic in nature. We previously reported an association between the ACE I/D polymorphism and salt sensitivity in a sample of patients with essential hypertension.[13] In the present study, we have extended the molecular genetic analysis of salt sensitivity to 8 polymorphisms of 6 genes of the RAAS: the ACE I/D, the angiotensinogen M235T, the angiotensin II receptor type 1 (AT_1) A1166C, the aldosterone synthase C-344T and intron 2 conversion, the MLR G3524C and A4582C, and the 11βHSD2 G534A in an extended sample of patients with essential hypertension.

Patients and Methods

Patients

Seventy-one patients with essential hypertension were consecutively recruited from the Hypertension Clinic of the Hospital Clínic, Barcelona (Spain). Secondary causes of hypertension were excluded after complete clinical, biochemical, and radiological examination. None of the patients included had renal impairment, papilledema, cardiac, coronary or cerebrovascular diseases, diabetes, or pregnancy. All the patients had ≥3 office BP measurements >140/90 mm Hg after 4 weeks of an unrestricted salt diet and without antihypertensive medication. Written informed consent was obtained from all the participants.

Diagnosis of Salt-Sensitive Hypertension

The methodology employed for the diagnosis of salt sensitivity has been published elsewhere.[14] Briefly, a low-salt diet containing 50 mmol sodium daily was administered to all participants for 14 days. This baseline diet was supplemented in a single-blind fashion by placebo tablets during the first period of 7 days (low-salt period) and by 12 tablets containing 17.8 mmol of sodium each, for the second period of 7 days (high-salt period), to achieve a total NaCl intake of 260 mmol daily. Compliance with diet was assessed by measuring the 24-hour urinary Na^+ excretion the last day of each period.

On the last day of each period, 24-hour ambulatory BP measurement was performed. Salt-sensitive hypertension was diagnosed, as previously reported,[14] when the increase in 24-hour mean BP from the low-salt period to the high-salt period was statistically significant ($P<0.05$).

RAAS Polymorphism Determination

Samples for DNA analysis were obtained from frozen peripheral leukocytes by standard procedures. The following polymorphisms were determined as previously published: ACE insertion/deletion (I/D),[15] angiotensinogen M235T,[16] AT_1 A1166C,[17] aldosterone synthase (CYP11B2) C-344T and gene conversion in intron 2,[18] and 11β HSD2 G534A.[19] In addition, 2 polymorphisms of the MLR gene were determined by restriction fragment length polymorphism . A G3514C transition in intron 4 was determined by polymerase chain reaction as reported[20] and specific restriction fragments were obtained with *Ban*II. A second polymorphism, A4582C (numbering according to Ludwig et al[20]), obtained from the Single Nucleotide Polymorphism Database (http://www.ncbi.nlm.nih.gov/SNP/) and located in the 3′UTR, was detected by amplification with the following primer pair: sense 5′-TTG GGA AAG CCT GCC TCG TT-3′ and antisense 5′-TCC TGC CAT GAT CTG TGC GTT-3′. Polymerase chain reaction was conducted in 25 μL volume containing 1.4 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 6% DMSO, 200 μmol/L of each dNTP, 1 μmol/L primers, 1U *Taq* polymerase, and 125 ng of genomic DNA. After an initial denaturation at 96°C for 5 minutes, thermocycling consisted of denaturation at 94°C for 30 seconds, annealing at 68°C for 30 seconds, and extension at 72°C for 30 seconds for 34 cycles, followed by a final extension for 5 minutes. Specific restriction fragments were obtained by digestion with *MspA1*I.

TABLE 1. Baseline Characteristics of Patients With Essential Hypertension Classified as Salt Sensitive or Salt Resistant

Parameter	Salt Sensitive (n=35)	Salt Resistant (n=36)	*P*
Age, y	47.9±2.1	46.4±2.2	0.620
Gender, m/f	21/14	21/15	1.000
Body mass index, kg/m^2	28.3±0.8	28.5±0.8	0.874
Systolic BP, mm Hg	150.9±4.0	151±2.8	0.978
Diastolic BP, mm Hg	92.9±3.3	92.6±1.9	0.936

Values are mean±SEM.

Statistical Analysis

Allele frequencies were estimated by the gene counting method, and Hardy-Weinberg equilibrium was tested by χ^2 test. One-way ANOVA was used to compare means among different genotype groups. The association of salt sensitivity with genotype distribution was tested by χ^2 test. To study the inheritance form of the polymorphic alleles that exhibited a significant relationship with salt sensitivity, association assuming a codominant effect, a dominant effect, and a recessive effect was calculated. The analysis of the interaction between genotypes was conducted by a logistic regression analysis. Values are expressed as mean±SEM, and $P<0.05$ was considered statistically significant.

Results

Following the criteria mentioned above, 35 patients (49%) were diagnosed as having salt-sensitive hypertension. Their mean increase in 24-hour BP was 9.9±1.2 mm Hg for systolic BP and 6.0±0.8 mm Hg for diastolic BP. The remaining 36 patients (51%) were considered as having salt-resistant hypertension. The BP increase with high salt intake in this group was −0.1±0.9 mm Hg for systolic BP and −1.1±0.6 mm Hg for diastolic BP. The baseline characteristics of salt-sensitive and salt-resistant patients are shown in Table 1. No differences were observed in terms of age, gender, body mass index, or baseline 24-hour BP. The genotype distribution exhibited a nonsignificant difference with that predicted by the Hardy-Weinberg equilibrium in all cases except in the MLR gene G3514C polymorphism ($P<0.001$).

Table 2 shows systolic and diastolic BP increases to high salt intake depending on different genotypes of the RAAS polymorphisms. As can be seen, and regarding the ACE I/D polymorphism, the increase in 24-hour BP in homozygous patients for the insertion allele (II) was significantly higher than that observed in homozygous patients for the deletion allele (DD). Heterozygous patients (DI) exhibited an intermediate response. On the other hand, there was a significant difference in systolic BP response to high salt intake between genotypes of the 11βHSD2 G534A polymorphism. The increase in GG patients was significantly higher than that observed in GA patients. Although some differences in

TABLE 2. BP Response to High Salt Intake Among the Genotypes of the ACE I/D and the 11βHSD2 G534A Polymorphisms

Genotype	Δ 24-Hour Systolic BP, mm Hg	*P*	Δ 24-Hour Diastolic BP, mm Hg	*P*
ACE I/D				
DD (n=21)	1.6±1.3		0.4±1.0	
DI (n=29)	5.2±1.5		2.6±1.1	
II (n=19)	8.3±2.1	0.031	4.9±1.1	0.012
AGT M235T				
MM (n=26)	4.9±1.7		2.4±1.2	
MT (n=27)	3.3±1.2		0.8±0.9	
TT (n=13)	6.8±2.9	0.435	5.5±1.9	0.056
AT_1 A1166C				
AA (n=32)	4.2±1.5		2.6±1.1	
AC (n=30)	5.8±1.5		2.7±1.0	
CC (n=5)	1.7±3.0	0.506	−0.9±2.1	0.429
AS C-344T				
CC (n=12)	2.2±1.4		1.1±1.3	
CT (n=23)	5.8±1.8		4.4±1.3	
TT (n=31)	4.7±1.6	0.464	1.6±1.0	0.144
AS intron 2 conversion				
cc (n=22)	3.4±1.7		0.8±1.2	
cw (n=26)	5.5±1.6		3.7±1.1	
ww (n=18)	5.0±2.0	0.649	2.9±1.4	0.238
11βHSD2 G534A				
GG (n=59)	5.4±1.0		2.8±7.3	
GA (n=7)	−1.3±3.5	0.039	−1.2±2.8	0.088
MLR G3514C				
GG (n=14)	3.7±1.4		1.5±0.8	
GC (n=12)	2.3±2.0		0.4±1.7	
CC (n=39)	6.0±1.4	0.331	3.5±1.0	0.233
MLR A4582C				
AA (n=6)	0.05±2.6		−0.09±2.1	
AC (n=33)	5.4±1.5		3.6±1.1	
CC (n=25)	5.1±1.6	0.333	2.1±1.0	0.181

Values are mean±SEM. AGT indicates angiotensinogen; AS, aldosterone synthase (for conversion polymorphism in intron 2, c indicates conversion and w indicates wild).

diastolic BP response were noted, they did not reach statistical significance.

The genotype distributions among the patients with essential hypertension when classified as salt sensitive or salt resistant are shown in Table 3. It can be appreciated that a significant difference in the distribution of genotypes was observed only with the ACE I/D polymorphism. The prevalence of salt-sensitive hypertension was also significantly different depending on the ACE genotype (19% for DD patients, 58.6% for DI patients, and 68.4% for II hypertensives; $P=0.003$). The association between the ACE I/D polymorphism and salt sensitivity was tested, assuming a dominant or a recessive model for the allele I. In the dominant model, the prevalence of salt-sensitive hypertension was compared in patients with both II and DI genotypes against patients with the DD genotype, showing a significant association (62.5% versus 19%, $P=0.001$). In contrast, when the prevalence of salt-sensitive hypertension in II patients was compared with DD and DI patients (recessive model for the I allele), the relative frequencies of salt sensitivity were not significantly different (68.4% versus 42%, $P=0.063$). The difference in genotype distribution of the 11βHSD2 polymorphism between salt-sensitive and salt-resistant hypertension was close to the significance level ($P=0.067$) (Table 3). The GA mutation in 11βHSD2 was associated with salt resistance because 85.7% of GA patients were salt resistant.

Table 4 shows the hormonal response to high salt intake analyzed by the ACE I/D and 11βHSD2 G534A genotypes. Although plasma renin activity (PRA) and aldosterone suppression following high-salt diet was higher in DD versus II patients, the difference was not statistically significant. On the contrary, patients with the 11βHSD2 GA genotype

TABLE 3. Genotype Distribution Among Patients With Essential Hypertension Classified as Salt Sensitive or Salt Resistant

Polymorphism	Salt Sensitive			Salt Resistant			*P*
ACE I/D	DD	DI	II	DD	DI	II	
	4 (11.8)	17 (50)	13 (38.2)	17 (48.6)	12 (34.3)	6 (17.1)	0.003
AGT M235T	MM	MT	TT	MM	MT	TT	
	10 (33.3)	12 (40.0)	8 (26.7)	16 (44.4)	15 (41.7)	5 (13.9)	0.391
AT1 A1166C	AA	AC	CC	AA	AC	CC	
	17 (53.1)	12 (37.5)	3 (9.4)	15 (42.9)	18 (51.4)	2 (5.7)	0.498
AS C-344T	CC	CT	TT	CC	CT	TT	
	4 (12.5)	15 (46.9)	13 (40.6)	8 (23.5)	8 (23.5)	18 (54)	0.112
AS conversion	cc	cw	ww	cc	cw	ww	
	8 (25)	16 (50)	8 (25)	14 (41.2)	10 (29.4)	10 (29.4)	0.203
11βHSD2	GG	GA		GG	GA		
G534A	30 (96.8)	1 (3.2)		29 (82.9)	6 (17.1)		0.067
MLR G3514C	GG	GC	CC	GG	GC	CC	
	5 (16.1)	5 (16.1)	21 (67.7)	9 (26.5)	7 (20.6)	18 (52.9)	0.456
MLR A4582C	AA	AC	CC	AA	AC	CC	
	1 (3.2)	17 (54.8)	13 (41.9)	5 (15.2)	16 (48.5)	12 (36.3)	0.262

Values are n (%). Abbreviations are the same as in Table 2.

(85.7% salt resistant), showed a significantly greater PRA suppression after a high-salt diet.

We tested a possible synergistic effect of the ACE I/D and 11βHSD2 G534A polymorphisms on BP variation to high salt intake by performing a paired genotype analysis. Table 5 shows that the paired genotype distribution was significantly different between salt-sensitive and salt-resistant patients. In addition, systolic BP response to high-salt diet was also significantly different among the paired genotypes, the effect apparently being due to ACE I/D polymorphism. Therefore, no synergistic effects were observed between these 2 polymorphisms. Similarly, a logistic regression analysis shows no significant synergistic effect of the remainder RAAS polymorphisms on the association between ACE gene I/D polymorphism and salt-sensitive hypertension (data not shown).

Discussion

In this study we demonstrate that out of 6 candidate genes of salt-sensitive hypertension tested, only 2 of them show an association with the BP response to high-salt diet in a sample of patients with essential hypertension. The ACE I/D polymorphism is associated with salt sensitivity in this sample of patients, because patients homozygous for the insertion allele (II) present a significantly higher BP response to high salt intake compared with that of patients homozygous for the deletion allele (D). Moreover, the prevalence of salt-sensitive hypertension in II (68%) and DI (59%) patients is significantly higher than that observed in DD patients (19%) and exhibits a dominant effect for the insertion allele. In addition, the systolic BP changes in response to high-salt diet were significantly different among the 11βHSD2 G534A genotypes, the GA patients being mostly salt resistant. In addition, GA patients show a significantly greater PRA suppression in response to high-salt diet compared with that of GG patients. A logistic regression analysis shows no significant synergistic effect of the remainder RAAS polymorphisms on the association between ACE gene I/D polymorphism and salt-sensitive hypertension. Age, gender, initial BP, and aldosterone did not significantly affect the BP change in response to salt. Although PRA change was different between salt- sensitive and salt-resistant patients, the inclusion of this parameter as a covariate did not affect the relationship between ACE genotype and systolic BP change.

TABLE 4. PRA and Plasma Aldosterone Response to High Salt Intake Among the Genotypes of the ACE I/D and the 11βHSD2 G534A Polymorphisms

Genotype	Decrease in PRA, ng · mL^{-1} · h^{-1}	Decrease in Plasma Aldosterone, pg/mL
ACE I/D		
DD (n=21)	0.22±0.1	9.0±1.8
DI (n=29)	0.32±0.09	9.9±2.1
II (n=19)	0.14±0.12	6.0±1.8
ANOVA *P*	0.383	0.370
11βHSD G534A		
GG (n=59)	0.20±0.06	8.3±1.2
GA (n=7)	0.63±0.22	11.4±3.3
t test *P*	0.018	0.415

The search for easily measurable markers of salt sensitivity in essential hypertension has motivated a great deal of research in the past several years. Studies in families suggest that salt sensitivity is genetically determined[1,2] and therefore justifies the search of genetic markers of this phenotype. Great success has been obtained in the molecular genetics of monogenic forms of hypertension, which are almost all salt sensitive.[12] On the contrary, the importance of genetic polymorphisms in the most common form of salt-sensitive hypertension (ie, essential hypertension) is not yet clear. Although the mechanisms responsible of salt-sensitive hypertension are

TABLE 5. Paired Genotype Distribution in Patients Classified as Salt Sensitive or Salt Resistant and BP Response to High Salt Intake Among Paired Genotypes of the ACE I/D and 11βHSD2 G534A Polymorphisms

Paired Genotypes	Salt Sensitive	Salt Resistant	Δ24-Hour Systolic BP, mm Hg*	Δ24-Hour Diastolic BP, mm Hg†
DD+GA (n=2)	0	5.9	6.1±3.4	−1.0±1.1
DI+GA (n=5)	6.7	8.8	−2.2±4.5	−0.14±4.1
II+GA (n=1)	0	2.9	−5.0	−2.0
DD+GG (n=18)	13.3	41.2	1.1±1.5	4.3±1.1
DI+GG (n=21)	40.0	26.5	6.3±1.6	3.0±1.3
II+GG (n=17)	40.0	14.7	8.3±2.1	4.7±1.3

Values for genotype distribution are %; values for BP are mean±SEM.
χ^2 P=0.037. *P=0.021, †P=0.219.

not fully elucidated, it is well known that the RAAS plays a central role.[3] Salt-sensitive patients display baseline RAAS suppression. Moreover, studies in families have demonstrated that the response of the RAAS to volume expansion and contraction is genetically determined,[2] and salt-sensitive patients display a blunted RAAS response to high salt intake, which is inversely correlated with the BP response.[5] This facts support the notion that patients with salt-sensitive hypertension may differ in genotype distribution of selected RAAS gene polymorphisms. The association of the II genotype with salt-sensitive hypertension confirms previous reports[13,21] and is in contrast with a report that, in contrast, evaluated salt sensitivity with different protocol.[22] If II patients have lower plasma ACE levels,[9] it could be speculated that this system could not be suppressed as efficiently as in DD patients when challenged with high-salt diet.

Mutations in the 11βHSD2 gene cause the apparent mineralocorticoid excess syndrome, a rare form of salt-sensitive hypertension.[12] One of the intermediary phenotype of this syndrome is the urinary excretion of a high ratio of cortisol/cortisone metabolites, which reflects a low activity of 11βHSD2.[12] Lovati et al[19] have found that healthy normotensive salt-sensitive subjects excreted far more cortisol metabolites relative to cortisone metabolites than did the salt-resistant subjects; they also found an association of salt sensitivity and low 11βHSD2 activity with a polymorphic marker in this gene. In our study using hypertensive patients, we did find a relation between systolic BP response to high salt and the G534A polymorphism. Moreover, the PRA response to high salt intake was also associated with this polymorphism, suggesting a participation of this gene in salt sensitivity in essential hypertension. We performed a paired genotype analysis of ACE I/D and 11βHSD2 G534A polymorphism and found that the association between salt sensitivity or BP response to high salt remained significant, but was not potentiated. Therefore, we cannot conclude that there was a synergistic effect between these 2 polymorphisms in determining salt sensitivity.

The angiotensinogen M235T polymorphism is associated with an increased risk of hypertension[7] and has also been evaluated in salt sensitivity, with controversial results. Although Hunt et al[23] reported that patients homozygous for the M allele had a lesser decrease in BP after mild sodium restriction than did hypertensives with TT or MT genotypes, Shorr et al[24] found no association of the M235T polymorphism and salt sensitivity in healthy male subjects. To our knowledge, the association between MLR gene variants and salt sensitivity in essential hypertension has not been previously reported. We assayed 2 different polymorphisms (intronic and coding) and found no association with salt sensitivity or with the BP response to high-salt diet in our sample of patients, as occurred with the AT_1 receptor A1166C polymorphism. Similarly, the negative finding with 2 aldosterone synthase gene polymorphisms in our patients is in agreement with a previous report performed in healthy male subjects.[25] Our power calculations with the data on systolic BP indicate that the total sample size required to detect a significant difference with an error <0.05 and a statistical power of 80% were 81, 285, 165, 221, 711, 28, 168, and 71 for the ACE, AGT, AT1, AS -344, AS conversion, 11βHSD2, MLR_{3514}, and MLR_{4582} polymorphisms, respectively.

Finally, we have examined a possible synergistic effect of these 8 polymorphisms on salt sensitivity by means of a logistic regression analysis. The successive addition of each polymorphism of the RAAS did not significantly affect the relation between the ACE I/D gene polymorphism and salt sensitivity.

In summary, of the 8 polymorphisms in 6 genes of the RAAS tested, only the ACE I/D and the 11βHSD2 G534A polymorphisms were significantly associated with the BP response to high salt in patients with essential hypertension. There was no synergistic effect between the different polymorphisms tested on salt sensitivity. Polymorphisms in other genes critical to the pathophysiology of salt sensitivity (ie, eicosanoids, adrenergic system) will need to be evaluated to gain insight into the genetic nature of salt sensitivity in essential hypertension.

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