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Synergistic effect of α-adducin and ACE genes causes blood pressure changes with body sodium and volume expansion

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Synergistic effect of α-adducin and ACE genes causes blood pressure changes with body sodium and volume expansion.

Background. The genetic dissection of a polygenic, multifactorial, quantitative disease such as arterial hypertension is hampered by a large environmental variance and by genetic heterogeneity.

Methods. To reduce the environmental variance, we measured the pressor response to a saline load (PRSL) and the basal plasma renin activity (PRA) under very controlled conditions in 145 essential hypertensive patients, as they may have the most direct clinical expression of the putative genetic alteration in renal Na handling and blood pressure (BP) regulation caused by the α-adducin and angiotensin-converting enzyme (ACE) polymorphism.

Results. PRSL was smaller in patients homozygous for the wild-type (Gly460) variant of α-adducin compared with that of patients bearing at least one copy of the 460Trp variant (2.5 ± 0.6 vs. 7.0 ± 0.9 mm Hg, $P = 0.0001$), whereas the ACE genotype was not associated with differences in PRSL. Both α-adducin and ACE affect PRA, with lower values correlated with the number of 460Trp or D alleles ($P = 0.019$ and 0.017, respectively). Most important, α-adducin and ACE interact epistatically in determining the PRSL, doubling the variance explained when epistasis is taken into account (variance from 7.7 to 15.5%).

Conclusion. These findings support the involvement of ACE and α-adducin in PRSL and PRA control, which are of paramount importance in setting the BP level and its response to therapy.

Primary or essential arterial hypertension, which affects about 20% of the adult population of Western countries, is a major risk factor for cardiac, renal, and cerebrovascular diseases. In spite of more than 40 years of research, the role of excessive Na intake in promoting hypertension and its cardiac complications is still under discussion. A series of observations has a consistent hypothesis that genetic mechanisms influence the relationship between Na intake and blood pressure (BP): (*1*) Not all humans have an increased BP when their Na intake is increased, and their genetic background influences this BP response [1]. (*2*) Salt sensitivity could be observed only in some, but not in all, animals in a colony of outbred, unrelated chimpanzees, the primate philogenetically closest to humans [2]. (*3*) Some rat strains develop genetic hypertension only if they ingest an excess of Na in their diets [3].

Although BP is influenced by many factors, pressure natriuresis plays an especially important role because it expresses the ability of the kidney to adjust Na excretion for a given perfusion pressure. It is the major determinant of the individual BP response to body Na changes. This key regulatory mechanism has to be reset in order to produce permanent BP changes [4]. Understanding its genetic control is of paramount importance in assessing the causes of primary hypertension [5]. Two independent considerations suggest that pressure natriuresis is influenced by many genes: (*1*) Multiple systems are involved, such as the sympathetic, renin-angiotensin, membrane transport, natriuretic factors systems, etc. (*2*) Being philogenetically ancient, protective homeostatic functions and genetic redundancy are likely to occur [6]. The relationship between systemic arterial pressure, renal perfusion pressure, and Na excretion developed at least 300- to 400-million years ago with teleosts [7]. To complicate the matter further, it should also be kept in mind that whenever an association is found between a gene polymorphism and a phenotype, the possibility that another gene mapping in close proximity to the one under study is the real causal gene cannot be excluded. Such a phenomenon could thus complicate gene-mapping studies. The demonstration of synergistic or interactive effects of two genes mapping on different chromosomes,

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both with a plausible role in causing the phenotype, would not only strongly reinforce their involvement, but also may help us understand the complexity of the genetic architecture of BP regulation [8].

We recently identified a polymorphism within the α-adducin gene, which is associated with hypertension in Milan hypertensive strain (MHS) rats and in a subset of humans [9, 10]. This association has been confirmed in other Caucasian [11] and Japanese populations [12, 13], but not by other researchers [14, 15]. Adducin is a cytoskeleton protein with very high degree of homology (94%) between rats and humans. The different α-adducin isoforms differentially affect the Na^+,K^+ pump activity on the basolateral tubular membrane, which is the driving force of overall tubular Na reabsorption [16]. This may explain why the patients with "hypertensive" (460Trp) α-adducin alleles are salt sensitive [10], have a less steep pressure natriuresis relationship [17], have an increased proximal tubular reabsorption [18], and have a larger BP fall after diuretic treatment and lower plasma renin activity (PRA) [10] when compared with patients homozygous for the "wild-type" (Gly460) adducin allele.

From an evolutionary point of view, the function of the renin-angiotensin system (RAS) has been to regulate blood volume and Na homeostasis since the emergence of amphibians, in which it is known that low nonpressor doses of angiotensin (Ang) increase tubular Na reabsorption without affecting the glomerular filtration rate [19]. An increased Ang II level shifts to the right and reduces the steepness of the slope of the pressure-natriuresis relationship, causing salt sensitivity, as shown with the infusion of subpressor doses of Ang II in the dog [20] and in transgenic (mRen-2)27 rats [21]. This mechanism is at least in part mediated by an Ang II-induced increase of the activity of the Na^+-H^+ exchanger [22] and of the Na^+,K^+-pump [23]. Therefore, we postulated that α-adducin and the various proteins of the RAS could interact (or cooperate) in the pressor response to changes in body Na. Among the possible candidate gene polymorphisms associated with the RAS, the angiotensin-converting enzyme (ACE) insertion/deletion (I/D) polymorphism was chosen because the D allele is associated with higher plasma and tissue ACE levels [24–27].

Therefore, the aim of our study was twofold: (*1*) to test whether the ACE gene I/D polymorphism affects the relationship between BP and acute changes of body Na content; and (*2*) to evaluate a possible synergistic effect or epistatic interaction between α-adducin polymorphism and ACE polymorphism on the pressure natriuresis relationship. The study was carried out in 145 untreated, essential hypertensive patients, with a constant Na intake of around 150 mEq a day for a month prior to the study in order to minimize all possible sources of variation that could hamper the detection of an influence of polymorphisms on the phenotypes under investigation.

METHODS

Patients

One hundred fifty-eight essential hypertensive patients were recruited from the "Outpatient Clinic for Hypertension" of San Raffaele Hospital of Milano (Italy). The following exclusion criteria were used: (*1*) history of myocardial infarction, congestive heart failure, stroke, creatinine clearance = 80 mL/min, diabetes mellitus, liver disease; (*2*) severe hypertension requiring immediate treatment; (*3*) women on oral contraceptives; and (*4*) individuals who were known to abuse drugs or alcohol. Inclusion criteria were: (*1*) a BP value greater than 145/95 but lower than 180/120 mm Hg in at least three consecutive visits at the outpatient clinic, and (*2*) a 24-hour ambulatory BP recording (Spacelabs 90207) with a mean daytime BP of greater than 140/90 mm Hg, or (*3*) pre-existing antihypertensive treatment. The exclusion criteria of the use of oral contraceptives led to the recruitment of far fewer women than men. Five patients were later excluded because of secondary hypertension (discussed later in this article). A large number of the patients studied had a recent diagnosis of hypertension or had never been treated before, so they could undergo the loading protocol within two to three weeks, provided that their usual Na intake was around 150 mmol Na per day, which is the average for the population living in the Milan area (checked by measuring 24-h urinary Na excretion). Otherwise, they were instructed to keep an average intake of 150 mmol Na/day for at least one month before Na loading. Of the 40 patients who had already been taking antihypertensive treatment, some had spontaneously discontinued it months before for various reasons, and others agreed to discontinue it for at least four months. In general, the therapy withdrawal occurred during the summer because of the spontaneous BP reduction in this season, with consequent easier removal of the therapy. We decided to perform such an unusually long period of therapy withdrawal to avoid any residual carryover effect of the previous treatment. This may be particularly important for patients taking diuretics or ACE inhibitors that may induce hypertrophy of the cells of the juxtaglomerular apparatus, which may take a long time to normalize. During the period of therapy withdrawal, the BP values were recorded at least every two weeks, and pharmacological treatment was resumed if the patient's diastolic BP exceeded 105 mm Hg. This led to the exclusion of five previously enrolled patients. Three patients did not show any BP increase after treatment was discontinued and are presently still in pharmacological washout; they were then excluded from the study. Also, the hypertensive patients in pharmacological washout were advised to keep a constant Na intake of approximately 150 mEq a day for at least one month before the study. All participants gave their

informed consent to the study. The present study is part of a larger study on the genetic mechanisms of hypertension that has been approved by the Ethical Committee of San Raffaele Hospital.

Acute salt-loading test

Patients were admitted to the hospital on Saturday morning, and they kept the same 150 mmol Na diet while in the hospital. They underwent clinical examination, routine biochemistry, and tests for the exclusion of secondary forms of hypertension between Saturday and Monday. The tests for the exclusion of secondary hypertension comprised renal function, plasma and urinary K, urinalysis, plasma renin activity (PRA), renal and suprarenal gland echography with color Doppler echography of the renal vasculature and, in case of any doubt, nuclear magnetic resonance (MR) angiography of the renal vasculature, MR of the suprarenal glands, and MR urography. Exclusion of secondary forms of hypertension was done during the hospital stay in order to have the patient accustomed to different clinical maneuvers and to minimize possible sources of environmental variation on the different patients. Secondary forms of hypertension were detected in five patients who were excluded from the study. PRA was measured on Saturday morning. Na loading was performed on Wednesday morning and consisted in the intravenous infusion of 2 L of saline (310 mmol NaCl) in two hours. BP was measured with a standardized procedure three times at five-minute intervals, before the beginning of loading, and three times, five minutes before, immediately after, and five minutes after the end of loading. BP values used in the analysis were the averages of the three readings. Urinary Na excretion during the loading was also measured.

Genotyping

The G460W polymorphism was investigated by polymerase chain reaction (PCR) amplification of genomic DNA followed by allele specific oligonucleotide hybridization as described by Cusi et al [10]. Genomic DNA (100 ng) was subjected to amplification using the following primers: forward 5′ GACAAGATGGCTGAACTC TGG 3′ and reverse 5′ AGTCTTCGACTTGGGACT GC 3′ in a total volume of 30 mL containing 10 mmol/L Tris-HCl (pH 8.8), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.1% Triton X-100, 15 pmol of each primer, 200 mmol/L of each deoxynucleotide triphosphate, and 1 U Taq polymerase. Amplification was carried out under the following conditions: 1 cycle of 4 minutes at 95°, 30 seconds at 57°, and 30 seconds at 72°, followed by 30 seconds at 95°, 20 seconds at 57°, and 30 seconds at 72°. Allele-specific oligonucleotide hybridization was as follows: the wild-type probe (for Gly460) was 5′ TTCTGCCCTTC CTC 3′, and the mutated probe (for Trp460) was 5′ TTCTGCCATTCCTC 3′. They were labeled with bacteriophage T4-polynucleotide kinase and used for hybridization.

The ID ACE gene polymorphism was detected, as described in Lindpainter et al [28]. Genomic DNA (100 ng) was amplified in a total volume of 30 mL containing 18 pmol of each primer, 670 mmol/L Tris-HCl, pH 8.8, 160 mmol/L $(NH_4)_2$ SO_4, 0.5% NP40, 50 mmol/L β mercaptoethanol, 1.3 mmol/L $MgCl_2$, 200 mmol/L of each deoxynucleotide triphosphate, and 1 U Taq polymerase. The sense oligonucleotide primer was 5′ GCCCTGCA GGTGTCTGCAGCATGT 3′, and the antisense primer was 5′ GGATGGCTCTCCCCGCCTTGTCTC 3′. Amplification was carried out under the following conditions: 35 cycles of 30 seconds at 94°, 45 seconds at 56°, and 2 minutes at 72°. As the D allele in heterozygous individuals was amplified preferentially, a second PCR step followed it for all samples genotyped as D/D in the first step, with primers that amplified a specific insertion sequence. PCR conditions were as before except for an annealing temperature of 67° and the following primers: sense oligonucleotide primer 5′ TGGGACCACAGCG CCCGCCACTAC 3′, antisense primer 5′ TCGCCAG CCCTCCCATGCCCATAA 3′.

Statistical analysis

Basic statistics. Differences between groups were analyzed with Student's *t*-test and one- or two-way analysis of variance (ANOVA) or analysis of covariance (ANCOVA, as specified in the **Results** section). To calculate the deviation from the Hardy–Weinberg equilibrium and sex ratios with χ^2, the SPSS (version 4.05) statistical software on an Apple Macintosh G3 personal computer was used. The significance level was set to $P < 0.05$. Data are expressed as mean values ± SEM.

Test for interaction. Two-locus model fitting and hypothesis testing was conducted via the least-squares regression analysis [29] with SAS statistical software on a workstation. The following simple linear model was fit to the data:

$$y_i = b_0 + \sum_{j=1}^{C} b_j^c c_{j,i} + b_{ACE} g_{ACE,i} + b_{G460} g_{G460,i} + b_{Int} g_{Int,i} + e_i$$

where y is a phenotype of interest gathered from individual i, b_0 is an intercept term, b_j^c is a coefficient measuring the effect of the *jth* [age, gender, body mass index (BMI), mean BP and basal urinary Na excretion] of the phenotype (C is the number of covariates included in the model). $c_{j,i}$ is a covariate measure on the *jth* covariate (dichotomous covariates were transformed into dummy variables; for example, gender was assigned as 0 = female, 1 = male); b_{ACE} and b_{G460} are regression coefficients measuring the effect of the ACE and α-adducin alleles, respectively; $g_{ACE,i}$ and $g_{G460,i}$ are genotype parameters assigned on the basis of genetic models. Thus, for additive allelic effects models, individuals were assigned a

Table 1. Baseline clinical characteristics of the 145 patients who underwent a sodium (Na)-sensitivity test

Variable	All samples	α-Adducin polymorphism			ACE polymorphism		
		Gly460Gly (N = 103)	Gly460Trp (N = 38)	Trp460Trp (N = 4)	I/I (N = 21)	I/D (N = 73)	D/D (N = 51)
Sex	127 M/18 F	92 M/11 F	31 M/7 F	4 M/0 F	19 M/2 F	64 M/9 F	44 M/7 F
BMI	25.6 ± 0.23	25.3 ± 0.26	26.2 ± 0.53	27.5 ± 1.54	24.7 ± 0.56	26.1 ± 0.36	25.2 ± 0.33
Age *years*	43.6 ± 0.73	42.8 ± 0.91	45.7 ± 1.20	46.2 ± 4.5	43.8 ± 2.23	43.8 ± 0.97	42.4 ± 1.28
Years of hypertension	4.9 ± 1.15	3.7 ± 0.53	4.2 ± 0.96	2.7 ± 1.20	3.5 ± 0.99	4.6 ± 0.74	2.8 ± 0.60
GFR *mL/min* (adj. to 1.73 m^2 BSA)	113.6 ± 1.9	112.9 ± 2.22	114.9 ± 3.89	118.4 ± 8.45	112.2 ± 4.29	112.7 ± 2.62	115.4 ± 3.43
Na serum *mEq/L*	141.0 ± 0.2	141.1 ± 0.2	140.9 ± 0.3	141.2 ± 0.8	140.7 ± 0.5	140.9 ± 0.3	141.3 ± 0.3
K serum *mEq/L*	4.19 ± 0.02	4.18 ± 0.03	4.19 ± 0.05	4.32 ± 0.018	4.1 ± 0.06	4.2 ± 0.04	4.2 ± 0.04
Ur Na excretion *mEq/24 h*	149.9 ± 4.39	154.4 ± 5.63	135.9 ± 5.69	169.0 ± 33.6	146.5 ± 2.91	154.2 ± 6.2	145.1 ± 7.31
Ur K excretion *mEq/24 h*	54.9 ± 1.41	54.1 ± 1.62	56.2 ± 2.96	61.7 ± 8.95	56.1 ± 3.95	56.0 ± 2.03	52.7 ± 2.22
Ur creatinine excretion *g/24 h* (adj. to 1.73 m^2 BSA)	1.12 ± 0.02	1.10 ± 0.02	1.15 ± 0.04	1.23 ± 0.13	1.12 ± 0.05	1.13 ± 0.03	1.10 ± 0.04
Systolic BP *mm Hg*	148.0 ± 1.18	147.8 ± 1.28	149.1 ± 2.71	140.6 ± 9.00	149.8 ± 2.91	145.6 ± 1.63	151.9 ± 1.99
Diastolic BP *mm Hg*	99.5 ± 0.72	99.4 ± 0.81	99.9 ± 1.60	97.2 ± 4.60	98.6 ± 1.84	99.04 ± 1.05	100.5 ± 1.18
Mean BP *mm Hg*	115.6 ± 0.82	115.5 ± 0.90	116.3 ± 1.91	111.7 ± 5.70	114.7 ± 2.09	114.5 ± 1.18	117.6 ± 1.35
PRA *ng/mL · h*	0.99 ± 0.06	1.08 ± 0.08	0.82 ± 0.08	0.49 ± 0.19	1.24 ± 0.19	1.05 ± 0.09	0.82 ± 0.08

Abbreviations are: BMI, body mass index; GFR, glomerular filtration rate; Ur, urinary; BP, blood pressure; PRA, plasma renin activity.

value of 0 if they were homozygous for one allele, 1/2 if heterozygous, and 1 if homozygous for the opposite allele. For dominant models, individuals were assigned a value of 0 if homozygous for one allele and 1 if heterozygous or homozygous for the opposite allele. b_{Int} is a coefficient measuring the effect of interaction between the two loci; $g_{Int.i}$ is a genotype interaction term created by simply multiplying relevant $g_{ACE.i}$ and $g_{G460.i}$ terms used in the model; and e_i is an error term. By fitting models with and without b_{Int} fixed to 0, hypothesis tests concerning the interaction effect could be pursued.

RESULTS

The polymorphisms were in the Hardy–Weinberg equilibrium for both the α-adducin (test for deviation, P = 0.97) and ACE (P = 0.82) loci. This should have ensured us that the results obtained were not due to sampling or stratification errors. Baseline clinical characteristics are summarized in Table 1 for the total sample and for subgroups divided according to α-adducin and ACE genotypes. All variables considered were similar among the different subgroups, except PRA, which decreased as a function of the number of copies of the 460Trp or D alleles (Fig. 1). The phenomenon is further analyzed in Table 2, in which the trend for PRA decrease appears to be additive between the 460Trp and D alleles. Unfortunately, this sample did not contain any Trp460Trp D/D individual, so that only eight of all nine possible genotypes were found because of the rare frequency of the Trp460Trp genotype. Although our patients received recommendations to maintain their Na intake at approximately 150 mmol/day, not all of them did so. In fact, the range of urinary Na excretion, although similar within the different genotypes, was relatively wide, with the extreme values of daily intake ranging from 65 to 131 mmol/day. However, the 95% CI was relatively small (141.2 to 158.6 mmol/day).

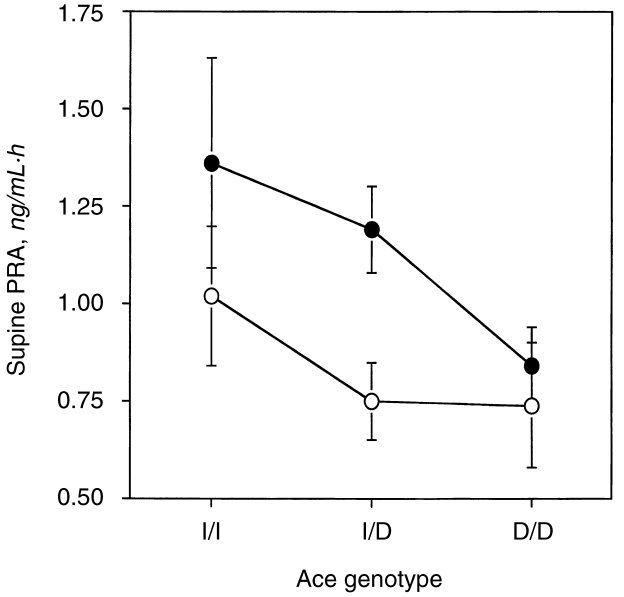

Fig. 1. Baseline supine plasma renin activity as a function of angiotensin converting enzyme (ACE) and the α-adducin genotype.

Analysis of genotype–phenotype associations for α-adducin and ACE independently

Blood pressure change according to the ACE genotype. The ACE genotype was not associated with any BP change after loading (I/I 3.5 ± 1.3; I/D 3.9 ± 0.7; D/D 3.8 ± 0.9 mm Hg, P = NS).

Blood pressure change according to the α-adducin genotype. Only four Trp460Trp homozygous hypertensives were found in the present sample. For this reason,

Table 2. Analysis of variance (ANOVA) with basal plasma renin activity (PRA) as the dependent variable and the genotype for angiotensin converting enzyme (ACE) and α-adducin as the independent variables

Source of variation	F	Significance of F	% Variance explained	Actual %
Main effects	3.675	0.007	Total variance	9.7
ACE genotype	4.232	0.017	Variance explained by ACE	4.4
α-Adducin genotype	4.104	0.019	Variance explained by α-adducin	5.3
Two-way interactions	0.392	0.759		

The model is highly significant, and the ACE and α-adducin genotypes contribute in a quantitatively similar way (around 5% each) to explain the total variance of PRA. The insignificant two-way interaction indicates that the rate of decline of PRA as a function of the number of D alleles is roughly parallel for each α-adducin genotype and vice-versa. In other words, the dose of D and 460Trp alleles contribute independently to decrease PRA.

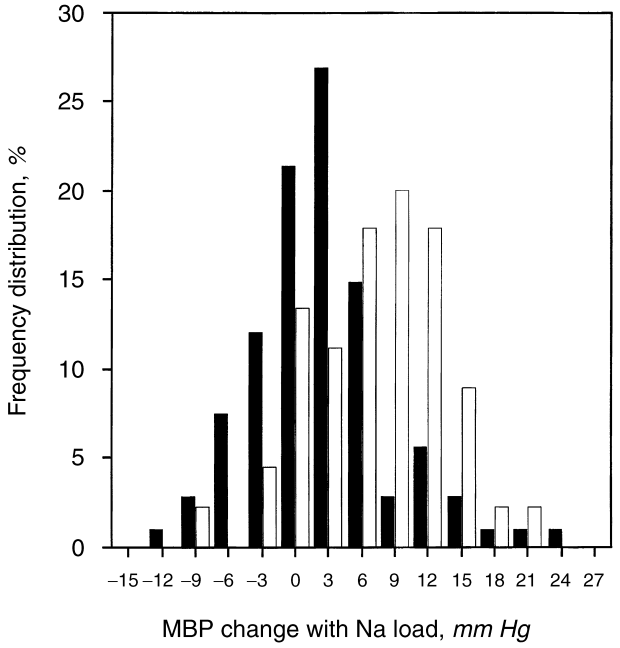

Fig. 2. Frequency distribution of mean blood pressure change with intravenous acute Na load according to the α-adducin genotype.

in the present analysis their BP changes with loading and depletion were counted with Gly460Trp heterozygotes. The average BP increase after loading was smaller in patients homozygous for the Gly460 allele than in those bearing at least one 460Trp allele (2.5 ± 0.6 vs. 7.0 ± 0.9 mm Hg, $P = 0.0001$), although there was a substantial overlap of the BP changes (Fig. 2).

Pressure natriuresis. Because of our experimental protocol, pressure natriuresis could only be evaluated by computing the ratio between the BP increase during loading as a function of the absolute amount of Na excreted during loading $\left(\frac{\Delta MBP}{NaUr}\right)$; however, taking into account its linearity [30], our estimate should be a reasonable approximation. The results are in substantial agreement with those of the absolute BP change after loading. No difference was seen among the ACE genotypes (I/I 0.07 ± 0.03 mmol Na/mm Hg; I/D 0.12 ± 0.02; D/D 0.09 ± 0.02, P = NS), while the hypertensive patients with the 460Trp allele of α-adducin had increases in their BP values that likely occurred in order to excrete the same quantity of Na (0.07 ± 0.02 mm Hg/mmol Na for Gly460Gly and 0.19 ± 0.016 for Gly460Trp + Trp460Trp, $P < 0.001$).

Genotype–phenotype associations: Analysis for α-adducin and ACE combined

When the effects of the two polymorphisms were analyzed together, ACE and α-adducin displayed a significant synergistic effect on the BP increase after loading and on the pressure-natriuresis relationship, since a large pressor effect of the ACE D allele could be seen only in the presence of the 469Trp allele (Fig. 3). Such an epistatic interaction was tested under different genetic assumptions. Analyses that assumed that the α-adducin 460Trp and ACE D alleles were codominant produced the best fit to the data. Table 3 reports the results of the model fitting, concentrating on the interaction terms for the additive contribution of either α-adducin and ACE to the model. Clearly, the model that included an interaction effect fitted the data substantially better than the model assuming that the loci have independent effects. In fact, the ACE locus effect is brought to light only in the context of its interaction with the 460Trp alleles, a phenomenon recently reviewed by Frankel and Schork [31]. Note that the inclusion of the interaction term into the model doubled the variance explained (from 7.7 to 15.5%). Similar results were found for $\frac{\Delta MBP}{NaUr}$. Also, in this case, the assumption of interactive allelic effects at each locus fits the data better, and the variance explained by the model that took into account the epistatic interaction again doubled (from 5.4 to 12.1%; Table 4).

DISCUSSION

The present results show that: (*1*) Patients possessing at least one copy of the 460Trp allele of α-adducin and the D/D genotype are the most responsive to an acute Na load. (*2*) ACE and α-adducin interact epistatically in the BP, increasing with the Na load; and (*3*) the number of copies of 460Trp and of the D allele independently

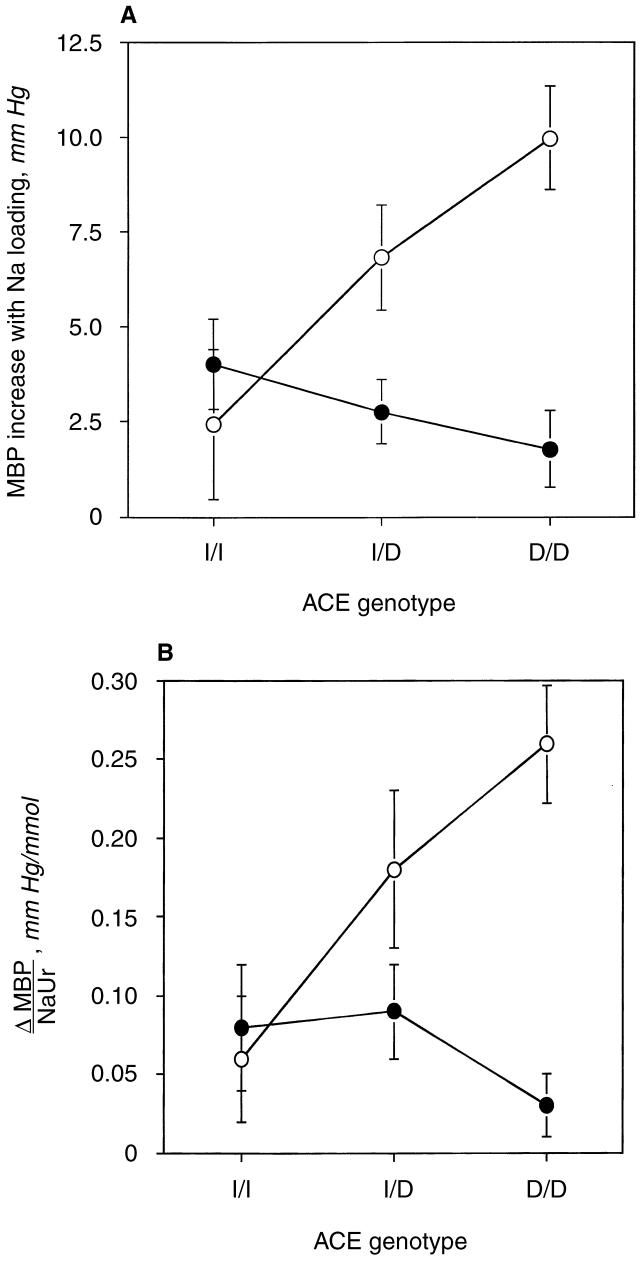

Fig. 3. (*A*) Change of mean blood pressure with intravenous acute Na load as a function of ACE and α-adducin genotype. (*B*) Pressure natriuresis $\left(\frac{\Delta MBP}{NaUr}\right)$ with intravenous acute Na load as a function of ACE and the α-adducin genotype.

Table 3. Regression model fitting results assuming independent effects and also taking into account the interaction effects between the loci for blood pressure increase after Na load

	Independent effects			Interaction effects		
ACE/adducin	F statistics	R^2	*P*	F statistics	R^2	*P*
Add/Add	5.95	7.73%	0.0033	8.16	15.49%	0.0001
Add/Dom	0.02	0.03%	0.9777	1.04	2.16%	0.3770
Add/Rec	7.73	9.82%	0.0006	8.13	14.76%	0.0001
Dom/Add	5.85	7.62%	0.0036	6.56	12.25%	0.0003
Dom/Dom	0.01	0.00%	0.9909	0.01	0.00%	0.9909
Dom/Rec	7.68	9.76%	0.0007	6.97	12.91%	0.0002
Rec/Add	5.99	7.79%	0.0032	7.26	13.38%	0.0001
Rec/Dom	0.06	0.08%	0.9450	1.04	2.17%	0.3755
Rec/Rec	7.77	9.86%	0.0006	7.13	13.17%	0.0002

Add, Dom, and Rec are the assumption of additivity, dominance, or recessivity, respectively, of the ACE/α-adducin alleles. The first entry gives the F-statistic, the second the variability explained by the model (R^2) and the third the *P* value associated with the F-statistic for the fit of the regression model.

Table 4. Regression model fitting results assuming independent effects and also taking into account the interaction effects between the loci for $\frac{\Delta MBP}{NaUr}$

	Independent effects			Interaction effects		
ACE/adducin	F statistics	R^2	*P*	F statistics	R^2	*P*
Add/Add	3.96	5.36%	0.0211	6.37	12.08%	0.0004
Add/Dom	0.14	0.2%	0.8658	0.39	0.84%	0.7588
Add/Rec	6.04	7.95%	0.0030	6.47	12.25%	0.0004
Dom/Add	4.04	5.45%	0.0198	5.57	10.74%	0.0012
Dom/Dom	0.39	0.55%	0.6805	0.39	0.55%	0.6805
Dom/Rec	6.17	8.10%	0.0027	6.05	11.55%	0.0007
Rec/Add	4.45	5.97%	0.0134	4.72	9.25%	0.0036
Rec/Dom	0.37	0.52%	0.6924	0.44	0.95%	0.7226
Rec/Rec	6.43	8.41%	0.0021	5.35	10.36%	0.0016

Add, Dom, and Rec are the assumption of additivity, dominance, or recessivity, respectively, of the ACE/α-adducin alleles. The first entry gives the F-statistic, the second the variability explained by the model (R^2) and the third the *P* value associated with the F-statistic for the fit of the regression model.

cause a decrease of PRA. Low PRA, which is commonly found in salt-sensitive individuals [1], is usually considered secondary to relative expansion of extracellular fluid volume. It is believed that salt-sensitive individuals have a relatively blunted RAS [32]. Changes in Na intake may affect the RAS, but this was not seen in our patients, because all of our patients had been on similar Na intake for at least one month before the study. The most likely explanation is that the D and the 460Trp alleles cause a larger renal Na retention for any given level of Na intake. Note that this also would have made them more responsive to the infused Na load.

Renal tubular cells transfected with the "hypertensive" rat α-adducin variant have faster activity of their Na^+,K^+ pump [16]. Humans and rats have different polymorphisms in the α-adducin subunit, but both interact with Na^+,K^+ pump, the "hypertensive" variants of both species have greater affinity for the Na^+,K^+ pump than the "normotensive" variants [33]. Taken together, all of these findings suggest that adducin could affect renal tubular reabsorption through the modulation of Na^+,K^+-pump activity.

The D allele of ACE gene is associated with a higher ACE concentration in several tissues as normal heart [25], lymphocytes [26], and proximal tubular cells [27]. Although direct evidence that its enzymatic activity

should produce locally acting Ang II is available only for vascular ACE [34], other circumstantial evidence suggests that ACE plays an important role in the regulation of BP, particularly in the experimental setup of gene targeting in mice, animals that express only serum ACE but lack tissue ACE and have a low BP [35]. Increased pressor responsiveness to exogenous Ang I and a higher generation of Ang II have been described in young normotensive humans with the D/D ACE genotype [36], which is consistent with the hypothesis that the D allele is associated with larger intrarenal generation of Ang II, a phenomenon that should promote renal Na retention. Others, who failed to confirm this finding [37], performed Ang I infusion under inhibition of renin, and it is not known how this maneuver affects the tissue ACE level in view of the contrasting effects of the salt diet on tissue and circulating RAS [38].

The question of why there is an additive effect between ACE and α-adducin for PRA in basal conditions and yet an epistatic interaction exists for the BP response to volume infusion and to $\frac{\Delta MBP}{NaUr}$ is an important one. In our study, each individual's PRA value may reflect a long-term intrinsic renal ability to regulate body Na and volumes. Differences in the time course of the resetting of the homeostatic regulation of BP/body Na relationship and in the nature of the environmental stimuli may account for the epistatic interaction observed with the Na infusion [39]. In fact, increased perfusion pressure generated by Na loading may depress tubular Na reabsorption by removing Na transport units from the luminal membranes, as well as Na^+,K^+ pump units from the basolateral membranes within minutes [40] (the rate of such removal can be controlled by α-adducin; Bertorello et al and Torielli et al, unpublished observations), while the rate of decrease of Ang II production may be smaller, and the time course of the reduction of Na reabsorption stimulated by locally-acting Ang II may be much slower. It should not be surprising to see different types of relationships among gene polymorphisms according to the duration of the study (in our case, at least one month of constant Na intake for PRA and two hours of saline infusion for pressure-natriuresis and acute BP changes).

Figure 2 confirms the well-known variability of the individual response to an expansion of body Na and volumes. As discussed elsewhere, the magnitude of the BP response to saline infusion depends on the algebraic sum of the depression of RAS, which, per se, may lower BP, and the positive pressure effect of the Na infusion [41]. We hypothesize that the degree of depression of tissue Ang II under saline infusion in D/D patients is less than that in I/I patients, and that this functional characteristic is unmasked and translated into a significant pressor effect only in the presence of the α-adducin 460Trp allele. It is interesting to note that in all cases, the BP change with load and the $\frac{\Delta MBP}{NaUr}$ relationship appeared to be linearly incremental by adding increasing doses of D alleles (from 0 to 2) in the different α-adducin genotypes, as summarized in Figure 3.

In spite of being a plausible candidate, the majority of the studies have not found an association between the ACE I/D polymorphism and hypertension until very recently, when large samples have been used [42–44]; however, these studies found only a relatively small significance level. The reason for the difficulty in finding positive results may be that in order to be clearly detectable, the effect of ACE genotype must be studied in a proper experimental setting that takes into account the appropriate combination of interacting alleles of other disease susceptibility gene(s). While recognizing that such an interaction without any a priori knowledge of the potential pathophysiological role of the interacting genes could be extremely difficult to prove, it was relatively easy in the present study, where an appropriate hypotheses and experimental setup had been used, and in which controls for environmental noise were devised.

As mentioned at the beginning of this article, the interaction between two candidate genes contributing to BP regulation and mapping on separate chromosomes provides very strong evidence for their causal involvement. Whether the long-term effect on BP levels caused by the ACE and α-adducin polymorphisms is synergistic, additive or negligible in unselected, and non-Na loaded hypertensive patients remains to be determined.

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REFERENCES

1. Weinberger MH: Salt sensitivity and blood pressure in humans. *Hypertension* 27(Pt 2):481–490, 1996
2. Denton D, Weisinger R, Mundy NI, Wickings EJ, Dixson A, Moisson P, Pingard AM, Shade R, Carey D, Ardaillou R, Paillard F, Chapman J, Thillet J, Michel JB: The effect of increased salt intake on blood pressure of chimpanzees. *Nat Med* 1:1009–1016, 1995
3. Dahl LK: Salt and blood pressure. *Lancet* 1:622–623, 1969
4. Hall JH, Mizelle L, Hildebrandt DA, Brands MW: Abnormal pressure natriuresis: A cause or a consequence of hypertension? *Hypertension* 15:547–559, 1990
5. Cusi D, Bianchi G: A primer on the genetics of hypertension. *Kidney Int* 54:328–342, 1998
6. Gibson TJ, Spring J: Genetic redundancy in vertebrates: Polyploidy and persistence of genes encoding multidomain proteins. *Trend Genet* 14:46–49, 1998

7. Damtzler WH: Comparative aspects of renal function, in *The Kidney: Physiology and Pathophysiology* (2nd ed), edited by Seldin DW, Giebisch G, New York, Raven Press, 1992, pp 885–942
8. Soubrier F, Lathrop GM: The genetic basis of hypertension. *Curr Opin Nephrol Hypertens* 4:177–181, 1995
9. Bianchi G, Tripodi G, Casari G, Salardi S, Barber BR, Garcia R, Leoni P, Torielli L, Cusi D, Ferrandi M, Pinna LA, Baralle FE, Ferrari P: Two point mutations within the adducin genes are involved in blood pressure variation. *Proc Natl Acad Sci USA* 91:3999–4003, 1994
10. Cusi D, Barlassina C, Azzani T, Casari G, Citterio L, Devoto M, Glorioso N, Lanzani C, Manunta P, Righetti M, Rivera R, Stella P, Troffa C, Zagato L, Bianchi G: α-Adducin polymorphism in primary hypertension: Linkage and association study; relationship to salt sensitivity. *Lancet* 349:1353–1357, 1997
11. Castellano M, Barlassina C, Muiesan M, Beschi M, Cinelli A, Rossi F, Rizzoni D, Cusi D, Agabiti-Rosei E: Alpha-adducin gene polymorphism and cardiovascular phenotypes in a general population. *J Hypertens* 15:1707–1710, 1997
12. Iwai N, Tamaki S, Nakamura Y, Kinoshita M: Polymorphism of alpha-adducin and hypertension. *Lancet* 350:369, 1997
13. Tamaki S, Iwai N, Tsujita Y, Nakamura Y, Kinoshita M: Polymorphism of alpha-adducin in Japanese patients with essential hypertension. *Hypertens Res* 21:29–32, 1998
14. Kato N, Sugiyama T, Nabika T, Morita H, Kurihara H, Yazaki Y, Yamori Y: Lack of association between the alpha-adducin locus and essential hypertension in the Japanese population. *Hypertension* 31:730–733, 1998
15. Ishikawa K, Katsuya T, Sato N, Nakata Y, Takami S, Takiuchi S, Fu Y, Higaki J, Ogihara T: No association between alpha-adducin 460 polymorphism and essential hypertension in a Japanese population. *Am J Hypertens* 11:502–506, 1998
16. Tripodi G, Valtorta F, Torielli L, Chieregatti E, Salardi S, Trusolino L, Menegon A, Ferrari P, Marchisio PC, Bianchi G: Hypertension-associated point mutations in the adducin alpha and beta subunits affect actin cytoskeleton and ion transport. *J Clin Invest* 97:2815–2822, 1996
17. Manunta P, Cusi D, Barlassina C, Righetti M, Lanzani C, D'Amico M, Buzzi L, Citterio L, Stella P, Rivera R, Bianchi G: Alpha-adducin polymorphisms and renal sodium handling in essential hypertensive patients. *Kidney Int* 53:1471–1478, 1998
18. Manunta P, Burnier M, D'Amico M, Buzzi L, Maillard M, Barlassina C, Lanella G, Cusi D, Bianchi G: Adducin polymorphism affects renal proximal tubule reabsorption in hypertension. *Hypertension* 33:694–697, 1999
19. Nashimura H: Endocrine control of renal handling of solutes and water in vertebrates. *Renal Physiol* 8:279–300, 1985
20. Hall JH, Mizelle HL, Woods LL: The renin-angiotensin system and long-term regulation of arterial pressure. *J Hypertens* 4:387–397, 1986
21. Lippoldt A, Gross V, Bohlender J, Ganten U, Luft FC: Lifelong angiotensin-converting enzyme inhibition, pressure natriuresis, and renin-angiotensin system gene expression in transgenic (mRen-2)27 rats. *J Am Soc Nephrol* 7:2119–2129, 1996
22. Cano A, Miller RT, Alpern RJ, Preisig PA: Angiotensin II stimulation of the Na-H antiport activity is cAMP independent in OKP cells. *Am J Physiol* 266:C1603–C1608, 1994
23. Aperia A, Holtback U, Syren ML, Svensson LB, Frickstedt J, Greengard P: Activation/deactivation of renal Na^+,K^+-ATPase: A final common pathway for regulation of natriuresis. *FASEB J* 8:436–439, 1994
24. Tiret L, Rigat B, Visvikis S, Breda C, Corvol P, Cambien F, Soubrier F: Evidence, from combined segregation and linkage analysis, that a variant of the angiotensin I-converting enzyme (ACE) gene controls plasma ACE levels. *Am J Hum Genet* 51:197–205, 1992
25. Danser AH, Schalekamp MA, Bax WA, van den Brink AM, Saxena PR, Riegger GA, Schunkert H: Angiotensin-converting enzyme in the human heart: Effect of the deletion/insertion polymorphism. *Circulation* 92:1387–1388, 1995
26. Costerousse O, Allegrini J, Lopez M, Alhenc-Gelas F: Angiotensin converting enzyme in human circulating mononuclear cells: Genetic polymorphism of expression inT-lymphocites. *Biochem J* 290:33–40, 1993
27. Mizuiri S, Yoshikawa H, Tanegashima M, Miyagi M, Kobayashi M, Sakai K, Hayashi I, Aikawa A, Ohara T, Hasegawa A: Renal ACE immunohistochemical localization in NIDDM patients with nephropathy. *Am J Kidney Dis* 31:301–307, 1998
28. Lindpainter K, Pfeffer MA, Kreutz R, Stampfer M, Grodstein F, Lamotte F, Buring J, Hennekens CK: A prospective evaluation of an angiotensin-converting-enzyme polymorphism and the risk of ischemic heart disease. *N Engl J Med* 332:706–711, 1995
29. Neter J, Waaserman W, Kutner MH: *Applied Linear Statistical Models.* Homewood, Richard D. Irwin, 1985
30. Kimura G, Brenner BM: Implications of the linear pressure-natriuresis relationship and importance of sodium sensitivity in hypertension. *J Hypertens* 15:1055–1061, 1997
31. Frankel WN, Schork NJ: Who's afraid of epistasis? *Nat Genet* 14:371–373, 1996
32. Weinberger MH, Stegner JE, Fineberg NS: A comparison of two tests for the assessment of blood pressure responses to sodium. *Am J Hypertens* 6:179–184, 1993
33. Ferrandi M, Salardi S, Tripodi G, Barassi P, Rivera R, Manunta P, Goldshlager R, Ferrari P, Bianchi G, Karlish JD: Interaction between adducin and Na,K-ATPase: Differential effects of hypertension related human and rat adducin polymorphism. *Am J Physiol* 277(4 Pt 2):H1338–H1349, 1999
34. Muller DN, Bohlender J, Hilgers KF, Dragun D, Costerousse O, Menard J, Luft CF: Vascular angiotensin-converting enzyme expression regulates local angiotensin II. *Hypertension* 29:98–104, 1997
35. Esther CR, Marino EM, Howard TE, Machaud A, Corvol P, Capecchi MR: The critical role of tissue angiotensin-converting enzyme as revealed by gene targeting in mice. *J Clin Invest* 99:2375–2385, 1997
36. Ueda S, Elliott H, Morton JJ, Connel JMC: Enhanced pressor response to angiotensin I in normotensive men with the deletion genotype (DD) for the angiotensin-converting enzyme. *Hypertension* 25:1266–1269, 1995
37. Laurichie ML, Azizi M, Guyene TT, Alhenc Gelas F, Menard J: Angiotensin-converting enzyme gene polymorphism has no influence on the circulating renin-angiotensin-aldosterone system or blood pressure in normotensive subjects. *Circulation* 91:2933–2942, 1995
38. Boddi M, Poggesi L, Coppo M, Zarone N, Sacchi S, Tania C, Neri Serneri GG: Human vascular renin-angiotensin system and its functional changes in relation to different sodium intakes. *Hypertension* 31:836–842, 1998
39. Guyton AC, Coleman TG, Cowley AV Jr, Scheel KW, Manning RD Jr, Norman RA Jr: Arterial pressure regulation. Overriding dominance of the kidneys in long-term regulation and in hypertension. *Am J Med* 52:584–594, 1972
40. Zhang Y, Magyar CE, Norian JM, Holstein-Ratholou NH, Mircheff AK, McDonough AA: Reversible effects of acute hypertension on proximal tubule sodium transporters. *Am J Physiol* 274:C1090–C1100, 1998
41. Cowley AW: Genetic and non genetic determinants of salt sensitivity and blood pressure. *Am J Clin Nutr* 65(Suppl):287S–593S, 1997
42. Julier C, Delepine M, Keavney B, Terwilliger J, Davis S, Weeks E, Bui T, Jeunemaitre X, Velho G, Froguel P, Ratcliffe P, Corvol P, Soubrier F, Lathrop GM: Genetic susceptibility for human familial hypertension in a region of homology with blood pressure linkage on rat chromosome 10. *Hum Mol Genet* 6:2077–2085, 1997
43. Fornage M, Amos CI, Kardia S, Sing CF, Turner ST, Boerwinkle E: Variation in the region of the angiotensin-converting enzyme gene influences interindividual differences in blood pressure levels in young white males. *Circulation* 97:1773–1779, 1998
44. O'Donnell CJ, Lindpaintner K, Larson MG, Rao VS, Ordovas JM, Schaefer EJ, Myers RH, Levy D: Evidence for association and genetic linkage of the angiotensin-converting enzyme locus with hypertension and blood pressure in men but not women in the Framingham heart study. *Circulation* 97:1766–1772, 1998