

Linkage but lack of association for blood pressure and the α -adducin locus in normotensive twins

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Background α -adducin is a cytoskeletal protein involved with sodium-pump activity in the renal tubule. The α -adducin gene locus has been linked to hypertension and a polymorphism identified which is associated with hypertension; however, the role of the α -adducin gene locus in normal blood pressure regulation is not defined. We performed a combined linkage and association study in normotensive monozygotic (MZ) and dizygotic (DZ) twins and their parents to address this issue.

Methods We studied 126 MZ and 70 DZ twin pairs and parents of DZ twins. Blood pressure values and responses to a cold pressor test were obtained. Cardiac dimensions were measured echocardiographically. Three microsatellites adjacent to the α -adducin gene were studied as well as the 460 Trp mutation in the α -adducin gene.

Results We obtained strong evidence for linkage ($P < 0.001$) between the α -adducin gene locus and systolic blood pressure. However, we were not able to associate the 460 Trp mutation with higher blood pressures, cold pressor responses or cardiac dimensions.

Introduction

Adducin is an α/β heterodimeric cytoskeletal protein present in many tissues, including the kidney [1]. The protein participates in the regulation of cell-signal transduction through changes in the actin cytoskeleton [2]. Alterations in adducin have been shown to influence the surface expression and maximum velocity of the sodium-potassium pump [2]. Known point mutations, one each in the α - and β -adducin subunits, account for up to 50% of the difference in blood pressure between the Milan hypertensive and normotensive rat strains [3]. An association between allelic markers close to the α -adducin gene locus and hypertension has been shown in man [4]. Recently, Cusi *et al.* [5] studied sibling pairs affected by hypertension. They were able to show that the α -adducin gene locus is linked to hypertension in these subjects. They then performed salt-sensitivity testing in hypertensive individuals with or without the 460 Trp α -adducin allele. The 460 Trp mutation was associated with hyper-

Conclusions The α -adducin gene locus is relevant to blood pressure regulation in normal subjects. Failure to find an association between higher blood pressures and the 460 Trp mutation suggests that this mutation may become important only when hypertension is triggered, or that other variations in α -adducin are present which have not yet been discovered. *J Hypertens* 1999, 17:1437–1441 © Lippincott Williams & Wilkins.

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tension. Persons heterozygous for the mutant Gly/Trp allele showed a greater decrease in blood pressure with acute volume contraction than subjects homozygous for the Gly/Gly wild-type allele. Furthermore, Gly/Trp individuals also displayed greater decreases in blood pressure with hydrochlorothiazide treatment, than wild-type homozygous hypertensive patients. These data implicate mutations in α -adducin as responsible for salt-sensitive hypertension in man. Hypertension affects 20% of the general population. Furthermore, over half of individuals reaching 70 years of age will develop hypertension [6]. Thus, identification of quantitative trait loci (QTL) for blood pressure regulation in normal subjects is of interest. There is precedence for such loci: a recent study has shown the Liddle hypertension gene locus to be an important QTL for blood pressure in normotensive persons [7]. We used a combined linkage and association approach to test the relevance of the α -adducin gene locus and its variation to blood pressure regulation in normal subjects.

Methods

We recruited 196 pairs of twins (126 monozygotic (MZ) and 70 dizygotic (DZ)) and the parents of the DZ twins by advertisement to participate in studies involving blood pressure regulation and cardiovascular phenotypes [8,9]. The subjects were all German Caucasians. They were recruited from various parts of Germany. The protocol was approved by Humboldt University's committee on the protection of human subjects, and written informed consent was obtained from all participants. Blood was obtained for the determination of zygosity and other molecular genetic studies from all the twins and the parents of the DZ twins. Each participant underwent a medical history and physical examination. None had hypertension or any other chronic medical illness. Blood pressure was measured after 5 min (two measurements, 1 min apart) with a standardized mercury sphygmomanometer in the sitting, standing and recumbent positions by a trained physician. The mean of the two measurements was used as the blood pressure.

M-mode and two-dimensional echocardiograms were recorded with patients in the left-lateral decubitus position. M-mode tracings that were guided two-dimensionally were recorded from the short parasternal axis at the chordal level between the free edges of the mitral leaflets at the tips of the papillary muscles. Only tracings with optimal visualization of left ventricular interfaces were used. In our echocardiographic laboratory, the range of variability of observations by a single reader is 0–1.5 mm for the left ventricular dimensions and 0–0.5 mm for the wall thickness. Interventricular septal thickness (IVS) and posterior-wall (PW) thickness were measured in all patients, and left ventricular dimensions were calculated by the Penn formula according to the guidelines of the American Society of Echocardiography [10].

For the linkage study, the DZ pairs were used as ordinary sibling pairs, but with the advantage of perfect age matching and reduced environmental variation affecting the phenotype. By genotyping the parents, we were able to perform an identity by descent (IBD) analysis. The power of the twin model in elucidation of complex genetic disease has recently been demonstrated by Martin *et al.* [11]. The novelty of utilizing combined linkage and association sibling pair analyses for quantitative traits, as we employ here, has recently been emphasized by Fulker *et al.* [12]. The MZ twins were used to estimate allele frequencies for the markers tested. The zygosity was verified with the use of five polymerase chain reaction-amplified microsatellite markers as described in detail elsewhere [13]. We examined three microsatellite markers at the α -adducin gene locus on chromosome 4 as described previously [4], namely D4S43, D4S95 and D4S228/E24. We then

used probes specific for the wild-type and mutated α -adducin gene sequences. The wild-type probe (for Gly 460) and the mutated probe for Trp 460 have been described previously [4]. The probes were labelled and hybridization studies of PCR products from the genomic DNA were performed to identify the point mutations in the α -adducin gene. A novel 5' nuclease assay was employed [14].

We assessed linkage for blood pressure as a continuous trait rather than differentiating between normotensive and hypertensive subjects [15]. Sibling pair analysis to determine linkage does not require the specification of a genetic model. The underlying trait can follow either Mendelian or non-Mendelian modes of inheritance. Analysis was done by using a structural equation modelling approach [16] as implemented in the MX package [17]. From the four alleles harboured by the parents for a given locus, each child randomly inherits two. Thus, a pair of siblings may have inherited either the same or different alleles. More specifically, they may share zero, one or two alleles identical by descent (IBD). If the locus under study is a QTL, phenotypic similarity of siblings (measured by the covariance) should increase with the number of alleles they share. Assuming no dominance effects, the total variance of the trait is due to the genetic effect of the QTL (Var_{qtl}), remaining additive genetic effects ($\text{Var}_{\text{addGen}}$) and environmental influences (Var_{env}):

$$\text{Var} = \text{Var}_{\text{qtl}} + \text{Var}_{\text{addGen}} + \text{Var}_{\text{env}}$$

Accordingly, the covariance of the three types of siblings as determined by their IBD status can be predicted as follows:

$$\text{CovIBD0} = 0.5\text{Var}_{\text{addGen}}$$

$$\text{CovIBD1} = 0.5\text{Var}_{\text{qtl}} + 0.5\text{Var}_{\text{addGen}}$$

$$\text{CovIBD2} = \text{Var}_{\text{qtl}} + 0.5\text{Var}_{\text{addGen}}$$

For linkage analysis, a model is specified estimating Var_{qtl} , $\text{Var}_{\text{addGen}}$ and Var_{env} so that the likelihood of the empirical variance–covariance matrix of the sibs, weighted by the probability of sharing zero, one or two alleles identical by descent, is maximized. For each sibling pair and each locus, the proportion of alleles IBD, based on parental genotypes, is calculated using a multi-point approach as implemented in MAPMAKER/SIBS [18]. To test for a QTL effect, the difference in model fit for models with and without a QTL effect is calculated as a χ^2 statistic. Since we used a candidate gene approach, we accepted $P < 0.01$ to test for significant linkage in accordance with the criteria defined by Lander and Kruglyak [19]. The high power of the variance–covariance-based analysis, nearly twofold

greater than the squared trait differences-based approach by the Haseman/Eston method, has been confirmed in a recent simulation study [20]. Thus, while significant linkage results obtained in smaller samples are still reliable, failure to detect linkage raises the issue of a lack of power and should not be interpreted as an exclusion.

Parameters of the quantitative genetic models were estimated by structural equation modelling using the MX program developed by Neale [17]. The variability of any given phenotype within a population can be broken down into genetic influences ($\text{Var}_{\text{addGen}}$), environmental influences shared by the twins within a family ($\text{Var}_{\text{sharedEnv}}$) and effects of random environment (Var_{env}):

$$\text{Var} = \text{Var}_{\text{addGen}} + \text{Var}_{\text{sharedEnv}} + \text{Var}_{\text{env}}$$

For MZ and DZ, the covariance of their phenotype is given by:

$$\text{CovMZ} = \text{Var}_{\text{addGen}} + \text{Var}_{\text{sharedEnv}}$$

$$\text{CovDZ} = 0.5\text{Var}_{\text{addGen}} + \text{Var}_{\text{sharedEnv}}$$

Heritability analysis in twin studies can estimate additive components of genetic variability as well as two environmental influences, shared and non-shared environmental influences [21]. These values estimate the relative amount of the variable's influence on inter-individual differences up to a sum of 1. Genetic as well as environmental effects were estimated by the best fitting model as selected by the χ^2 value. Statistical analysis was conducted using the SPSS program (SPSS Inc., Chicago, Illinois, USA). Adjustment of blood pressure values for sex and age was done by multiple linear regression with the unstandardized residuals as the corrected phenotypes.

Finally, we performed a bivariate analysis to determine the degree to which systolic and diastolic blood pressure are influenced by different genes or different sets of genes [21]. This analysis relies on maximum likelihood estimates of genetic influences.

Results

Demographic data, blood pressure values and heredity estimates of blood pressure and echocardiographic variables in 252 MZ and 140 DZ twins are given in Table 1. There were no significant differences between MZ and DZ twins for any of the variables examined. Systolic and diastolic blood pressure were heritable. The heritability estimates for systolic blood pressure were about double those for diastolic blood pressure. The echocardiographic parameters also demonstrated strong evidence of heritability.

Table 1 Demographic data, phenotypic values (mean \pm SD), heredity estimates (α^2) and correlations (r) for monozygotic (MZ) and dizygotic (DZ) twin subjects

	MZ twins	DZ twins	α^2 (rMZ/rDZ)
<i>n</i>	252	140	
Age (years)	29 \pm 12	31 \pm 12	
Sex (M/F)	52/148	85/47	
Height (cm)	169 \pm 8	170 \pm 8	
Weight (kg)	65 \pm 11	67 \pm 12	
BMI (kg/m ²)	22.4 \pm 3.5	22.8 \pm 3.4	
Systolic BP sitting (mmHg)	118 \pm 11	118 \pm 10	0.81 (0.81/0.31)
Diastolic BP sitting (mmHg)	69 \pm 9	71 \pm 8	0.41 (0.80/0.59)
Posterior wall thickness (mm)	8.7 \pm 1.6	8.6 \pm 1.6	0.26 (0.48/0.26)
Septum (mm)	8.9 \pm 1.7	8.8 \pm 1.6	0.37 (0.64/0.37)

Table 2 shows the results of linkage analysis for the tested loci in the DZ twins using systolic and diastolic blood pressure in the sitting, standing and recumbent position as the phenotype. The blood pressure values are corrected for age and gender, although uncorrected values gave similar results. We found strong evidence for linkage of systolic blood pressure in the sitting and standing positions to the α -adducin gene locus. For systolic blood pressure in the recumbent position, the results are suggestive for linkage. These results establish the α -adducin gene locus as a QTL for systolic blood pressure. We were not able to show such evidence for linkage to diastolic blood pressure.

We tested the hypothesis that systolic blood pressure and diastolic blood pressure may be influenced by different genes. The extent to which both systolic and diastolic blood pressure are influenced by the same genes was measured by the genetic correlation (r_g). For blood pressure measured in the sitting position, the value was $r_g = 0.53$. To test the significance of the genetic correlation, submodels of the bivariate analysis were calculated setting either the shared or specific genetic influences to zero. Both submodels resulted in a significant loss of fit ($P < 0.01$), confirming the importance of both the shared as well as the specific genetic influences.

Table 2 Results of linkage analysis. The difference between structural equation modelling with/without quantitative trait loci (QTL) effect and corresponding P values for a QTL effect are given

	χ^2 model difference	P value
Systolic BP recumbent	5.78	0.016
Systolic BP sitting	6.94	0.008
Systolic BP standing	14.41	<0.001
Diastolic BP recumbent	0.00	NS
Diastolic BP sitting	2.31	NS
Diastolic BP standing	0.00	NS
Systolic BP increase (cold pressor test)	0.00	NS
Diastolic BP increase (cold pressor test)	0.00	NS
Septum thickness	0.00	NS
Posterior wall thickness	0.00	NS
Left ventricular mass	0.00	NS

BP, blood pressure; NS, not significant.

Table 3 shows the results of the association analysis. For this analysis, the DZ twins and one of the MZ twins of each pair were genotyped, 266 subjects in all. We found no association between wild-type Gly/Gly homozygosity, Gly/Trp heterozygosity or Trp/Trp homozygosity on blood pressure or heart size. Hardy-Weinberg criteria were fulfilled.

Discussion

The important finding in this study is that the α -adducin gene locus was linked to systolic blood pressure in young healthy individuals. To our knowledge, this is the first observation to document the importance of α -adducin to blood pressure regulation prior to the development of hypertension. The result implies that variability in the α -adducin gene must exist, which influences blood pressure. Interestingly, we could not show that the 460 Trp polymorphism contributed to this variability in normal individuals. Our findings are confined to systolic blood pressure. We found no linkage to diastolic blood pressure. There are several possible explanations. The genes for regulatory systems influencing systolic and diastolic blood pressure may not invariably be the same, a hypothesis we tested. Systolic blood pressure may be more accurately measured. Finally, the robustness of genetic variance on blood pressure in our study was almost twice as great for systolic as for diastolic blood pressure.

Systolic and diastolic blood pressure may well be influenced by different genes. For instance, systolic blood pressure may be more dependent on aortic elasticity than diastolic blood pressure, which in turn might be more dependent on peripheral vascular resistance. To test the hypothesis that systolic and diastolic blood pressure are affected by different sets of genes, as well as pleiotropic genes, we performed a bivariate heritability analysis according to the methods of Neale and Cardon [21]. Based on the variance-covariance matrix of the two traits in MZ and DZ twins, both genetic

influences shared by the traits and specific genetic influences are estimated. The extent to which both systolic and diastolic blood pressure are influenced by the same genes can then be measured by the genetic correlation. Our results indicates that about 50% of the genetic influences on blood pressure affect both systolic and diastolic blood pressure, an example of pleiotropy. The remaining genetic influences are specific to either systolic blood pressure or diastolic blood pressure. To test the significance of the genetic correlation, submodels of the bivariate analysis are calculated, setting either the shared or specific genetic influences to zero. Both submodels confirmed the importance of both the shared and the specific genetic influences in our subjects.

We believe our approach of identifying linkage in normotensive DZ twin subjects and their parents is a uniquely powerful method to establish the relevance of genes regulating blood pressure in normal people. In an earlier study, we found strong evidence for linkage of the IGF-1 and renin gene loci to blood pressure [22]. The subsequent step is to identify all the genetic variants in these candidate genes so that these variants can be retested in terms of an association study in the same subjects. There is precedence for this approach. We recently employed multiplex sequencing to identify all variants in the β -2 adrenergic receptor gene in the normotensive offspring of hypertensive parents from the Bergen Blood Pressure Study [23]. Four mutations causing amino acid substitutions were found, which were in linkage disequilibrium with each other. The complexity of the resulting haplotype analysis is a harbinger of the future difficulties geneticists will encounter when considering the effects of multiple genes and their haplotypes simultaneously.

Our failure to find an association between variances at the 460 Trp site and blood pressure in these normal subjects by no means distracts from the potential importance of this polymorphism. Some of our subjects may develop hypertension at a later date, at which time such association may become apparent. The association was particularly prominent in terms of responses to volume loading and contraction, and in terms of blood pressure decreases with diuretic therapy in the studies of Cusi *et al.* [5] and subsequent observations by Manunta *et al.* [24]. We did not examine salt sensitivity or resistance in this study. Conceivably, had we performed salt sensitivity testing in these normal subjects, we might have identified allelic association. We did, however, examine the blood pressure responses to a stressor impulse, the cold pressor test. In an earlier study [8], we showed that this response is also influenced by genetic variance, but that different genes were likely to be responsible. Thus, our failure to find an association between the α -adducin gene locus and

Table 3 Effects of the α -adducin polymorphism. The dizygotic twins and one of the monozygotic twins from each pair were genotyped

	Gly/Gly	Gly/Trp	Trp/Trp
<i>n</i>	200	63	3
Systolic BP recumbent	126 ± 14	127 ± 15	121 ± 12
Systolic BP sitting	124 ± 13	124 ± 15	119 ± 13
Systolic BP standing	123 ± 14	124 ± 15	115 ± 7
Diastolic BP recumbent	71 ± 11	71 ± 11	63 ± 7
Diastolic BP sitting	73 ± 10	74 ± 11	63 ± 9
Diastolic BP standing	80 ± 9	80 ± 9	74 ± 12
Systolic BP increase (cold pressor test)	11 ± 11	10 ± 10	12 ± 12
Diastolic BP increase (cold pressor test)	8 ± 7	7 ± 6	7 ± 6
Septum thickness	8.7 ± 1.5	9.0 ± 1.6	8.5 ± 0.7
Posterior wall thickness	8.5 ± 1.6	8.8 ± 1.6	8.5 ± 0.7
Left ventricular mass	168 ± 54	175 ± 57	166 ± 15

There were no significant differences between groups. BP, blood pressure.

this response may very well be predicated on the fact that different genes are involved. Kamitani *et al.* [25] searched for association between the 460 Trp mutation and high or low blood pressure from a Scottish cohort. The subjects were selected in terms of being offspring from hypertensive or normotensive parents. No overall association between the α -adducin genotypes and blood pressure variation could be found in that study.

α -adducin and its relevance to blood pressure regulation was first identified in the Milan genetic strain of hypertensive rats. To our knowledge, α -adducin is the first and only novel rodent discovery relevant to human hypertension. In the Milan strain, hypertension can be transplanted with the kidney, suggesting that α -adducin expression in that organ is of primary importance, as opposed to α -adducin expression elsewhere [26]. Cross-immunization of cytoskeleton proteins between the Milan hypertensive and normotensive rat strains showed immunochemical differences in rat adducin [27]. Such studies have not been performed with human adducin and the relevance of the 460 Trp mutation to differences in the human protein structure is not known for certain. Possibly, the 460 Trp mutation is in linkage disequilibrium with some other as yet undiscovered mutation. To elucidate this issue, the entire gene will have to be sequenced in a population such as ours, so that all genetic variants are known, as was done in the subjects of the Bergen Blood Pressure Study [23].

In the study by Cusi *et al.* [5], large numbers of mutant 460 Trp/Trp homozygous subjects were present who were not hypertensive. This fact indicates that interaction with other genes and environmental factors must be present to develop the hypertensive phenotype. Possibly, α -adducin gains in importance when some other triggering factor exercises an effect. Such a mechanism might also explain why we were unable to find an association with the 460 Trp mutation and higher blood pressure in these normal subjects. In conclusion, our data support the notion that the α -adducin gene locus is important to blood pressure regulation in normal man. Sequencing the entire gene in this subject cohort and in others will elucidate all α -adducin polymorphisms influencing blood pressure. Such an effort in this and other genes will be necessary to further elucidate the molecular genetics of blood pressure regulation in normal persons and essential hypertension.

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