

Twin Studies in the Analysis of Minor Physiological Differences Between Individuals

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Key Words

Genetics • Twins • Channels • Blood pressure • Linkage • Association • Hypertrophy • Kinase • Protein kinases • Polymorphism

Abstract

To test the hypothesis that the serum and glucocorticoid regulated kinase (SGK1) is of relevance to blood pressure in man, we recruited monozygotic (MZ) (126 pairs) and dizygotic (DZ) (70 pairs) normotensive twin subjects and parents of DZ twins. Blood pressure was measured in a controlled fashion recumbent, sitting, and upright. We documented genetic variance on blood pressure in all positions. We then relied on microsatellite markers at the SGK1 gene locus (D6S472, D6S 1038, and D6S270) and two single nucleotide polymorphisms within the SGK1 gene. We found significant linkage of the SGK1 gene locus to diastolic blood pressure ($p < 0.0002$) and suggestive evidence for linkage for systolic blood pressure ($p < 0.04$), documenting the locus as a QTL for blood pressure. We next performed association, using all DZ twins and an MZ member from each pair. We found significant associations between both SNP variants and blood pressure, as well as a significant interaction between the SNPs enhancing the effect. This

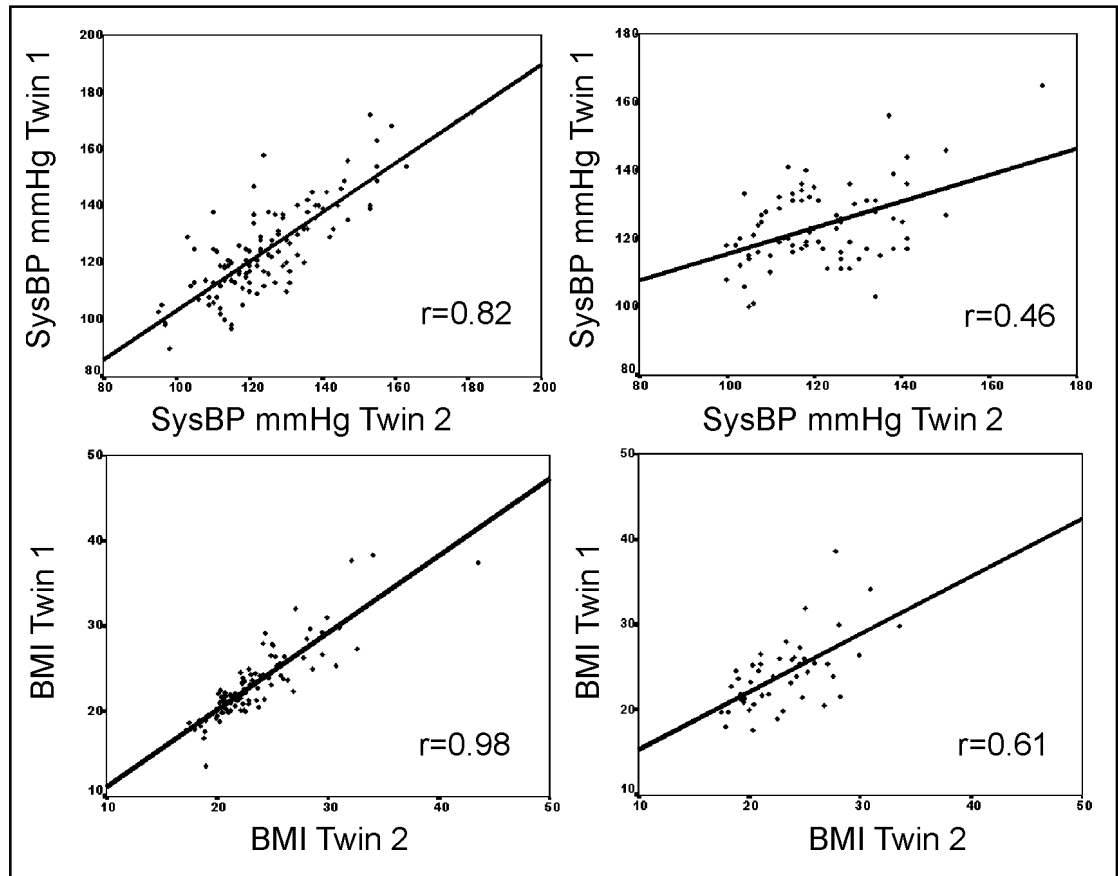
combined effect of the polymorphisms was confirmed in an independent sample of 260 young normotensive men. These data, coupled with our earlier observations linking the insulin-like growth factor-1 gene locus to blood pressure lead us to conclude that the SGK1 gene is relevant to blood pressure regulation and probably to hypertension in man.

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Introduction

In earlier reviews, we pointed out the utility of the twin model in the genetics of quantitative traits [1, 2]. The method was first given a sound foundation by Siemens, who showed that twin subjects are by nature very generous persons and can be recruited from schools, universities, and through public advertisement. He also developed an effective method of zygosity testing to distinguish monozygotic (MZ) from dizygotic (DZ) twins. Finally, he was the first to propose comparing traits in MZ and DZ twins, reasoning that MZ twins share all their genes in common, while DZ twins share half their genes being no more similar genetically than other sib-

Fig. 1. Within-pair resemblance in MZ (left panels) and DZ (right panels) twin pairs for systolic blood pressure (upper panels) and body mass index (lower panels). The correlation values r for MZ twins are around 0.8 while the r values for DZ twins are around 0.4. The similarity for MZ twins is significantly greater than for DZ twins.



lings [3]. However, like MZ twins, DZ twins are born at the same time and are likely to be exposed to similar environmental conditions. The twin method is based on the fact that MZ twins arise from division of one zygote. Thus, they must be genetically identical or “clones” of one another. Any phenotypic difference between the two must be caused by environmental influences. Environment in this view encompasses anything that is not genetically determined. The method assumes that DZ twins are influenced by largely similar environmental differences as MZ twins, but have only half their genes in common by descent. This fact makes them ideal control subjects. Many phenotypes can be investigated with the twin model. Relevant to hypertension is the documentation of the genetic contribution to renal function and electrolyte balance [4, 5]. We have primarily focused on blood pressure regulation and baroreflex function. Both are influenced by genes that express channel proteins or that influence channel function. Thus far, our phenotypes have not included channel function of individual cells, although such functions could be readily assessed.

Twin analysis

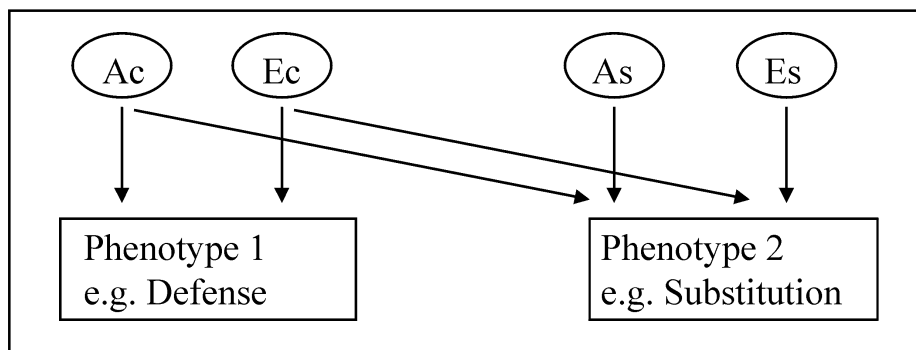
Examples of twin analyses from the Berlin cohort pertinent to cardiovascular disease are shown in figure 1. For instance, the correlation for blood pressure in MZ twins is greater than for DZ twins (upper panel). The same applies for body mass index (lower panel). In the Berlin twin studies, parameters of the quantitative genetic models were estimated by path analysis techniques using MX by Neale [6]. Analogous to a regression analysis, the variability of any given phenotype (Var) within a population can be separated into genetic influences ($\text{Var}_{\text{addGen}}$), environmental influences shared by the twins within a family ($\text{Var}_{\text{comEnv}}$), and random environment (Var_{Env}):

$$\text{Var} = \text{Var}_{\text{addGen}} + \text{Var}_{\text{comEnv}} + \text{Var}_{\text{Env}}$$

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

$$\begin{aligned} \text{Cov}_{\text{MZ}} &= \text{Var}_{\text{addGen}} + \text{Var}_{\text{comEnv}} \quad \text{and} \\ \text{Cov}_{\text{DZ}} &= 0.5\text{Var}_{\text{addGen}} + \text{Var}_{\text{comEnv}} \end{aligned}$$

Fig. 2. Bivariate path model that separates the phenotypic variance and covariance for two given phenotypes (for instance defense and substitution) into additive genetic (Ac) and environmental (Ec) contributions that are common to both phenotypes, and factors that are specific to one phenotype only, additive genetic (As) and environmental (Es) contributions.



Path analysis in twin studies can estimate additive components of genetic variability (estimated as a^2) as well as two environmental influences, shared (c^2), and nonshared environmental influences (e^2) [7]. For the purpose of this discussion, we are ignoring nonadditive genetic effects of dominance or epistasis. These values estimate the relative amount of the variable's influence on interindividual differences up to a sum of one. Genetic and environmental effects were estimated by the best fitting model as selected by the χ^2 value.

An example of path analysis as applied to blood pressure during resting state and cold pressor testing is shown in figure 2. The path model includes two sets of genes, one influencing both resting and stress values (A_a), the second set of genes influencing only the stress values (A_b), two sets of shared environmental factors (C_a and C_b), and two sets of nonshared environmental factors (E_a and E_b) respectively. In addition to comparing the absolute levels of blood pressure at rest and during cold pressor, the bivariate model was applied to the resting level of blood pressure and to the blood pressure responses (ΔBP) to stress as well. Since the correlation between blood pressure at rest and blood pressure during cold pressor stress in our subjects was not significant ($p > 0.05$), we used the absolute differences (blood pressure with cold pressor - blood pressure at rest) as a ΔBP value, rather than residualized change scores. This type of model tests the hypothesis that blood pressure at rest and the ΔBP value with cold pressor stress share genetic variability.

QTL linkage

Twin studies lend themselves to molecular genetic investigations both in terms of association and linkage analyses. The power of the twin model in elucidating complex genetic disease was emphasized by Martin et

al. [8] For linkage studies, the DZ pairs are studied as sib-pairs with the advantage of perfect age matching and reduced environmental variation affecting the phenotype. The MZ twins are used to estimate allele frequencies for the markers tested. The zygosity is verified with microsatellite markers [9]. We have generally assessed linkage for continuous traits rather than differentiating between qualitative categories [10]. Sib-pair analysis to determine linkage does not require the specification of a genetic model. We use the structural equation modeling approach [11] as implemented in the MX-package [6]. Shown in figure 3 is an example of two parents harboring distinct alleles at a given locus; each child randomly inherits two. The children may share zero, one, or two alleles identical by descent (IBD). If the locus under study is a QTL, phenotypic similarity of sibs (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}), the remaining additive genetic effects ($\text{Var}_{\text{addGen}}$), and the environmental influences (Var_{Env}):

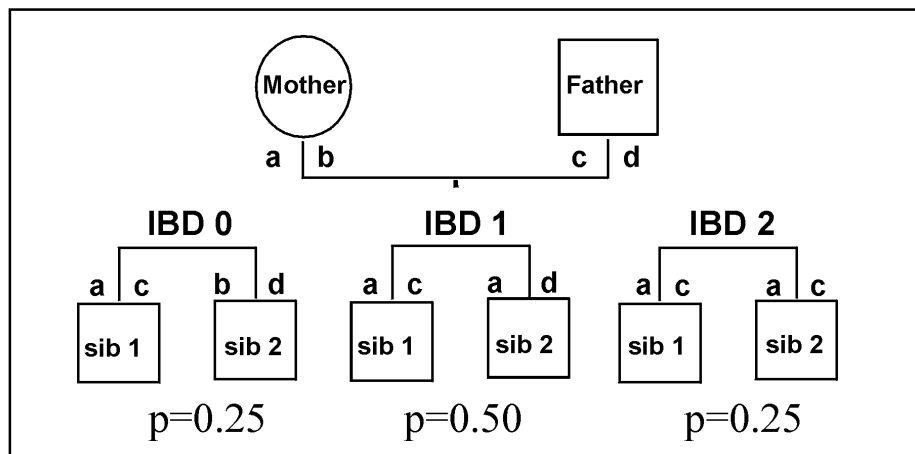
$$\text{Var} = \text{Var}_{\text{qtl}} + \text{Var}_{\text{addGen}} + \text{Var}_{\text{Env}}$$

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

$$\begin{aligned} \text{COV}_{\text{IBD0}} &= 0.5 \text{Var}_{\text{addGen}} \\ \text{COV}_{\text{IBD1}} &= 0.5 \text{Var}_{\text{qtl}} + 0.5 \text{Var}_{\text{addGen}} \\ \text{COV}_{\text{IBD02}} &= \text{Var}_{\text{qtl}} + 0.5 \text{Var}_{\text{addGen}} \end{aligned}$$

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 IBD, is maximized. For each sib pair and each locus, the proportion of alleles IBD based on parental

Fig. 3. Affected sib-pair approach is shown in which the possible distribution of marker alleles in the offspring are given provided the parents are ab and cd respectively. 25% of the offspring share no alleles in common, 25% share both alleles in common, and 50% share 1 allele in common. Linkage is indicated if the allele sharing for IBD1 or IBD2 are increased. For the QTL analysis, the phenotypic similarity of sibs (measured by the covariance) should increase with the number of alleles they share.



genotypes is calculated using a multipoint approach as implemented in MAPMAKER/SIBS [12]. To test for a QTL effect, the absolute difference in model fit for models with and without a QTL effect is calculated as a χ^2 statistic. Since we usually use a candidate gene approach, we accept $p < 0.01$ to test for significant linkage in accordance with the criteria defined by Lander and Kruglyak [13]. We also used the more established single-point Haseman-Elston (H/E) approach, as implemented in the SIBPAL program in the SAGE package [14]. To test for linkage, a linear regression analysis is carried out with the squared trait difference as the dependent variable and IBD as the independent variable. The high power of the variance-covariance based analysis, nearly twofold compared to the squared trait differences based approach by the H/E method, has been confirmed in a recent simulation study [15]. 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.

Examples of linkage studies we have done in terms of blood pressure include evidence from the epithelial sodium channel β and γ subunit loci, the locus for insulin-like growth factor-1 (IGF-1) [16], and the locus for the serum and glucocorticoid-regulated kinase [17]. The demographic data from our twin studies is given in table 1. Some less robust results were also identified for the angiotensin II AT1 receptor locus and the renin locus. The linkage analysis showed significant or suggestive linkage of the SGK1 gene locus to systolic and diastolic blood pressure in all positions. These data provide evidence implicating the SGK1 gene locus in blood pressure regulation.

Association and other analyses

We have found utility in combining linkage and association analyses. With linkage, we addressed the question "where is it?". With association (for example case-control studies), we can formulate the question "what is it?", in terms of candidate genes residing at the locus of interest. Single nucleotide polymorphisms (SNP) are useful for this purpose. Furthermore, the availability of family structures (individuals and parents) allows for transmission disequilibrium tests and other haplotype-sharing approaches. An example of this approach is our study of SNPs in the SGK1 gene [17]. The genomic organization of SGK1 has been described earlier [18]. Information can be obtained at gene no 00000118515, www.ensembl.org. We first searched public databases to identify SNPs in SGK1; however, most SNPs were either erroneous or were not sufficiently polymorphic for our purposes. A SNP in Exon 8 (C→T) that we identified from the NCBI SNP database proved satisfactory (rs 1057293). We eventually relied on direct sequencing and found a second SNP 551 base-pairs away located in the donor site (Intron 6) of Exon 7 (T→C). These two single SNPs, Intron 6 (T→C) and Exon 8 (C→T) were analyzed.

Table 4 shows the results for systolic and diastolic blood pressure in the recumbent, sitting, and standing positions and the SNP genotypes. The allele frequencies for the Exon 8 SNP were C 91%, T 9%. Those for the Intron 6 SNP were T 79%, C 21%. Hardy-Weinberg equilibrium was maintained for both polymorphisms. The blood pressure values in all positions were very similar. Homozygous and heterozygous carriers of the Exon 8

Table 1. Demographic data, phenotypic values (mean±SD), heredity estimates(a^2) and correlations (r) for MZ and DZ.

	MZ twins	DZ twins	a^2 (r_{MZ}/r_{DZ})
N	200	132	
Age, y	29±12	31±12	
Sex M/F	52/148	85/47	
Height, cm	169±8	170±8	
Weight, kg	65±11	67±12	
BMI, kg/m ²	22.4±3.5	22.8±3.4	
Systolic BP sitting, mm Hg	118±11	118±10	0.81 (0.81/0.31)
Diastolic BP sitting, mm Hg	69±9	71±8	0.41 (0.80/0.59)
Posterior wall thickness, mm	8.7±1.6	8.6±1.6	0.26 (0.48/0.26)
Septum, mm	8.9±1.7	8.8±1.6	0.37 (0.64/0.37)

Table 2. Linkage analysis for blood pressure (corrected for age and gender) in the sitting, standing, and recumbent position. For systolic blood pressure results of both H/E analysis and SEM analysis are given.

Locus	H/E		SEM				
	sys BP sitting	sys BP sitting	sys BP standing	sys BP recumbent	dia BP sitting	dia BP standing	dia BP recumbent
IGF1	0.00001	0.00005	0.0002	0.004	0.9	0.9	0.3
Chromosome 12							
Liddle	0.012	0.026	0.010	0.9	0.9	0.9	0.9
Chromosome 16							
AT ₁ receptor	0.027	0.0008	0.32	0.0026	0.9	0.9	0.21
Chromosome 3							
Renin	0.046	0.0009	0.000001	0.0018	0.0036	0.009	0.008
Chromosome 1							

alleles (CC or CT) did not differ from each other while showing higher systolic and diastolic blood pressure values than homozygous Exon 8 TT subjects. The results for the Intron 6 SNP were less consistent; however, homozygous CC persons in general had higher blood pressure values than homozygous TT or heterozygous TC persons.

Table 3 gives the association statistics for the twin subjects. For the phenotypes systolic and diastolic blood pressure, the Exon 8 SNP was invariably significant except for diastolic blood pressure in the recumbent position. The results for Intron 6 SNP were similar for systolic blood pressure. The SNPs were in strong linkage disequilibrium, as shown in table 4. Everyone homozygous for Exon 8 TT was also homozygous for Intron 6 CC; however, the converse was not invariably the case. The highest blood pressure values were observed for subjects carrying the increasing alleles for both SNPs. For association studies, a confirmatory cohort is highly desirable to verify the results. We tested this association in an independent, 260 subject confirmation sample and

found a significant effect of both polymorphisms on resting systolic blood pressure. Again the blood pressure increasing effect of Intron 6 CC was compensated by the lowering effect of Exon 8 TT in subjects homozygous for both polymorphisms. For diastolic blood pressure no association could be detected.

Discussion

Our findings underscore the utility of studies in normal MZ and DZ twin subjects. Including the parents of DZ twins facilitates linkage studies as we have shown here. For association, multiple SNPs are more helpful than solely relying on one or two variants. Had we relied only on a single SNP, our analysis would have been less informative. By analyzing two SNPs we were able to document an interaction which solidifies our results. A similar but more comprehensive study was recently reported by Zhu et al. [19]. They performed a linkage and association analysis of angiotensin I-converting enzyme

Table 3. Mean blood pressure (mm Hg) values, left ventricular enddiastolic and endsystolic diameter (LVEDD and LVESD, mm), posterior wall and septum thickness (mm) \pm SD for SNPs in Exon 8 (C \rightarrow T) and Intron 6 (T \rightarrow C). p-values are given for the simultaneous ANOVA of both polymorphisms.

Phenotype	Intron 6 TT (n=174)	Intron 6 CT (n=72)	Intron 6 CC (n=18)	Intron 6 TT/CT (n=246)	p CC vs. TT/CT
BP_level	-0.04 \pm 1	0.10 \pm 1	-0.39 \pm 1	-0.02 \pm 1	0.07
SystBP recumbent	125 \pm 15	125 \pm 18	132 \pm 14	125 \pm 16	0.05
DiastBP recumbent	70 \pm 10	72 \pm 13	74 \pm 12	71 \pm 11	n.s.
SystBP sitting	124 \pm 14	123 \pm 15	129 \pm 13	124 \pm 14	0.05
DiastBP sitting	72 \pm 10	74 \pm 10	79 \pm 9	73 \pm 10	n.s.
SystBP standing	123 \pm 15	123 \pm 14	129 \pm 13	123 \pm 15	0.05
DiastBP standing	79 \pm 10	81 \pm 10	84 \pm 8	80 \pm 10	n.s.
LVEDD	48 \pm 4	48 \pm 4	51 \pm 4	48 \pm 4	0.01
LVESD	29 \pm 4	28 \pm 4	29 \pm 3	29 \pm 4	n.s.
Posterior wall	8.8 \pm 1.7	8.3 \pm 1.7	9.2 \pm 1.4	8.6 \pm 1.7	n.s.
Septum	9.0 \pm 1.7	8.5 \pm 1.8	9.5 \pm 1.3	8.8 \pm 1.8	n.s.

Phenotype	Exon 8 CC (n=223)	Exon 8 CT (n=36)	Exon 8 TT (n=5)	Exon 8 CC/CT (n=259)	p TT vs. CC/CT
BP_level	-0.01 \pm 1	-0.10 \pm 1	0.29 \pm 1	-0.04 \pm 1	0.05
SystBP recumbent	125 \pm 16	128 \pm 18	119 \pm 6	126 \pm 16	0.01
DiastBP recumbent	71 \pm 10	72 \pm 13	67 \pm 10	71 \pm 11	n.s.
SystBP sitting	124 \pm 14	125 \pm 17	117 \pm 6	124 \pm 14	0.05
DiastBP sitting	73 \pm 10	74 \pm 11	72 \pm 9	73 \pm 10	0.05
SystBP standing	123 \pm 14	126 \pm 16	119 \pm 8	123 \pm 15	0.01
DiastBP standing	80 \pm 10	82 \pm 11	78 \pm 8	80 \pm 10	0.05
LVEDD	48 \pm 4	49 \pm 3	52 \pm 6	48 \pm 4	n.s.
LVESD	29 \pm 4	29 \pm 3	31 \pm 2	29 \pm 4	n.s.
Posterior wall	8.7 \pm 1.6	8.3 \pm 1.9	10.0 \pm 2.0	8.6 \pm 1.7	n.s.
Septum	8.9 \pm 1.7	8.4 \pm 1.9	10.0 \pm 2.6	8.9 \pm 1.7	n.s.

Table 4. Linkage disequilibrium between the two polymorphisms in the twin sample (chi² test p < 0.01).

SNPs	Intron 6 TT	Intron 6 TC	Intron 6 CC
Exon 8 CC	172 (64%)	48 (19%)	3 (1%)
Exon 8 CT	2 (1%)	24 (10%)	10 (4%)
Exon 8 TT	0 (0%)	0 (0%)	5 (2%)

(ACE) gene polymorphisms with ACE concentrations and blood pressure in 1,343 Nigerians from 332 families. Most of the polymorphisms in the ACE gene were significantly associated with ACE levels. The two most highly associated polymorphisms accounted for 6% and 19% of the variance in ACE respectively. The authors then used a two-locus additive model and with an additive-x-additive interaction of these polymorphisms were able to explain most of the ACE variation in this region. They also showed an association with systolic and diastolic blood pressure when the two-locus additive model was used.

SGK1 and indeed all of the aldosterone-induced gene transcripts are attractive candidate genes for blood pressure regulation and particularly for hypertension. The small G protein K-Ras2A and the aldosterone-inducible protein SGK1 both appear to increase ENaC activity. K-Ras2A is a channel activating aldosterone-induced protein that is involved in GTP-induced increases in channel activity and requires prenylation [20] Activation of Ras upregulates and disruption of Ras prenylation downregulates SGK1 transcription, pointing to interaction of the two signaling molecules. Once expressed, SGK1 requires activation that can be accomplished by

insulin and insulin-like growth factor IGF1 [20, 21]. The signaling of insulin and IGF1 to SGK1 involves PI3-kinase and subsequent activation of the serine/threonine kinases PDK1 or PDK2. ENaC upregulation by mineralocorticoids requires activation through the PI3 kinase pathway, which mediates the stimulation of renal Na⁺ excretion by insulin and IGF1 [22]. Thus, SGK1 may participate in the signaling of IGF1-induced alterations of renal Na⁺ transport and blood pressure. Acromegaly, which leads to enhanced release of IGF1, is a well-known cause of hypertension and the IGF1 gene locus, as we review here, demonstrates linkage to blood pressure in twins [23, 24].

Further compelling evidence linking SGK1 to hypertension comes from SGK1 gene-disrupted mice. Wulff et al. recently demonstrated that these mice have a major difficulty maintaining sodium balance because of ENaC dysregulation and defective distal tubular sodium reabsorption [25]. Furthermore, Yun et al. recently showed that SGK1 is important in regulating and stimulating the ROMK potassium channel. This channel is also important to sodium reabsorption via ENaC. We believe that the SGK1 gene is an excellent candidate gene for blood pressure regulation and hypertension.

Limitations of our study are the fact that the linkage data are derived from a relatively small number of DZ twins and their parents and that the gene locus has not been robustly linked to blood pressure regulation and hypertension in an earlier study. An example for the reproducibility of positive linkage findings in relatively small samples would be the gene(s) responsible for Liddle's syndrome [27]. The locus was linked to blood pressure in the general population in parallel on the basis of family studies and in the twin study from our group as shown here. Careful physiological hypotheses, replication, and concordant evidence from linkage and association can add credibility to genetic studies. We provide evidence for linkage between the SGK1 gene locus and systolic and diastolic blood pressure under several different conditions. We performed an association analysis of two SNPs in the SGK1 gene and found that both were associated with systolic and diastolic blood pressure. We were then able to show a significant interaction, which enhanced the effect. We conclude that SGK1 may be important to blood pressure regulation and hypertension.

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