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Loss-of-Function Polymorphism of the Human Kallikrein Gene with Reduced Urinary Kallikrein Activity

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Abstract. Kallikrein is synthesized in the distal tubules and produces kinins, which are involved in the regulation of vascular tone in the kidney. Urinary kallikrein activity has been reported to be partly inherited and to be reduced in essential hypertension. In a systematic search for molecular variants of the human kallikrein gene, nine single-nucleotide polymorphisms were identified. Five of those polymorphisms, including two nonsynonymous substitutions in exon 3, *i.e.*, Arg53His (allelic frequency in Caucasian subjects, 0.03) and Gln121Glu (allelic frequency, 0.33), were studied in a normotensive group and two independent hypertensive groups for which 24-h urinary kallikrein activity had been measured. A significant decrease in urinary kallikrein activity was observed for the subjects who were heterozygous for the Arg53His polymorphism, compared with the other subjects. This finding was consistent in the two hypertensive groups and was observed with several kallikrein enzymatic assays. The Gln121Glu polymorphism and the other polymorphisms were not associated with changes in urinary kallikrein activity. None of the polymorphisms was associated with hypertension. Recombinant kallikrein variants were synthesized and enzymatically characterized, using native kininogen and kininogen-derived synthetic peptide substrates. No important effect was observed after Gln121 mutation, but there was a major decrease in enzyme activity when Arg53 was replaced by histidine. A model of kallikrein derived from crystallographic data suggested that Arg53 can affect substrate binding. The identification of a subset of subjects with genetically reduced kallikrein activity as a result of an amino acid mutation could facilitate analysis of the role of the kallikrein-kinin system in renal and vascular diseases.

Tissue kallikrein (hK1) is the major kinin-forming enzyme in the kidney (1,2). Kallikrein is synthesized in the distal tubules and released in urine and the peritubular interstitium. Kinins have potent endothelium-mediated vasodilatory and antithrombotic properties, and several studies have documented the role of the kallikrein-kinin system, and its interaction with the renin-angiotensin system, in the regulation of the medullary and papillary circulation (1,3,4). The level of urinary kallikrein excretion reflects the renal synthesis of the enzyme. Urinary kallikrein activity is influenced by genetic factors and by dietary sodium intake. Two studies have documented familial patterns of urinary kallikrein activity (5,6). On the basis of studies of large Utah pedigrees, Berry *et al.* (6) have suggested that a major gene effect accounts for one-half of the variance of the trait, with a dominant allele being associated with high urinary kallikrein activity and a reduced risk of hypertension. Urinary kallikrein excretion is reduced in renal diseases and in essential hypertension (1,7). An association between BP and a kallikrein gene polymorphism has been observed in rat strains (8), but no relationship between kallikrein gene polymorphism and urinary kallikrein activity levels or BP status has been established for human subjects.

The kallikrein gene (*hKLK1* gene) is located on chromosome 19 (19q13.2-q13.4), together with several homologous genes coding for non-kinin-forming serine proteases or unidentified protein products (9). The *hKLK1* gene contains five exons, spanning >5.2 kb, and codes for an inactive prokallikrein form that is activated by intracellular proteolysis of a short amino-terminal peptide (10). Several polymorphisms of the *hKLK1* gene have been identified. In addition to a microsatellite marker at the *hKLK1* gene locus (11), a complex multiallelic polymorphism has been identified in the 5'-flanking region of the gene, with two alleles displaying lower *in vitro* promoter activity (12). Berge and Berg have also described a *Taq*I polymorphism (13) and three additional diallelic polymorphisms, including one leading to an amino acid substitution (Arg53His) (14).

The identification of single-nucleotide polymorphisms (SNP) of candidate genes provides powerful tools for the identification of genetic factors associated with quantitative traits and complex diseases (15). To study the role of the kallikrein gene in the genetic polymorphism of urinary kal-

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likrein activity and to identify new genomic markers useful for assessment of the role of kallikrein in disease, we conducted a systematic search for SNP. Nine were identified, and the five most frequently observed were tested for association with urinary kallikrein activity and hypertension. Two nonsynonymous SNP, namely Arg53His and Gln121Glu, were further studied by *in vitro* synthesis of recombinant kallikrein variants. We demonstrate that one, the R53H mutation, dramatically reduces the activity of the enzyme and is associated with a reduction in the level of urinary kallikrein activity.

Materials and Methods

Subjects

Genomic DNA analysis was first performed for a sample of 46 Caucasian and 40 Afro-Caribbean hypertensive subjects who were randomly selected from the HYPERGENE data set of hypertensive families (16). The most relevant alleles were then genotyped in several groups of Caucasian subjects, as follows: (*1*) a group of 255 normotensive subjects matched for age and gender with the hypertensive subjects from the HYPERGENE database, whose main characteristics were described in previous case-control studies (17); (*2*) a group of 74 unrelated individuals with essential hypertension who were involved in a clinical trial in which baseline 24-h urinary kallikrein excretion, with an unrestricted sodium diet, was measured (18); and (*3*) a group of 164 hypertensive subjects participating in a multicenter European and North American investigation (the HyperPATH group) intended to evaluate the genetic features of human hypertension, for whom several intermediate phenotypes, including 24-h urinary kallikrein excretion, were assessed with 1-wk high- and low-salt diets (19,20). The institutional review board of each center approved the studies, and all subjects provided written informed consent for the genetic analysis.

Identification of Molecular Variants and Genotyping

The search for single-strand conformational polymorphisms was performed with genomic DNA, as described previously (21). The primers were designed to correspond to the 5'-flanking region of the *hKLK1* gene from position −361 to position +1 (10) and to the sequences upstream and downstream of each of the five exons, to display the slightest degree of similarity with the *hKLK2* gene (9) and to provide amplification products of <250 bp (Table 1). Each sample was subjected to electrophoresis under two different conditions, first in a 0.5× mutation detection enhancement gel (BioWhittaker, Rockland, ME) prepared in 0.6× TBE (1× TBE contains 90 mM Tris-borate, pH 7.8, and 2 mM ethylenediaminetetraacetate) and developed at room temperature at 400 V for 14 to 20 h and then in a 5% polyacrylamide gel (49:1, polyacrylamide/methylenebisacrylamide) prepared in 0.5× TBE and developed at 4°C at 15 W for 3 to 4 h. Direct sequencing of electrophoretic variants was performed by using the dideoxy chain-termination method, with an ABI 377 analyzer (PE Applied Biosystems, Foster City, CA).

Genotyping was performed by using the allele-specific oligonucleotide hybridization procedure. Specific oligomers corresponding to the wild-type and mutated alleles are presented in Table 1. Numbering was performed with reference to the transcription initiation site (10) for the promoter region and with reference to the first base of the initiator methionine codon for the exonic sequence. Ambiguous results were reanalyzed by using a PCR-restriction fragment length polymorphism method with restriction enzymes corresponding to the polymorphic sites, *i.e.*, *Fun4H*I for the C−128G polymorphism, *Mnl*I for the C405T mutation, and *Bstn*I for the C433G (Q121E) mutation.

Table 1. Primers derived from the *hKLK1* gene sequence used for polymorphism identification and genotyping

Primers	5′-Sequence-3′
SSCP[a]	
5′-F_1	GGTCACTGATTCCTCCGTCTTC
5′-R_1	TTGCCCCCCCGCCTCCGATC
5′-F_2	GCGTGATCCAGGGCCTGCAG
5′-R_2	CAGGGACAGGGCGAGCGAC
exon 1-F	GTTCCCCAGTTCCTCCACCTG
exon 1-R	GGCCCGTTCCCCCTCCCAC
exon 2-F	CCTGGCCTCTCCTGCCAACC
exon 2-R	CTCCTCCCAGACCCCAGGCC
exon 3-F_1	CCTTCCCGTCTTCTCATCCC
exon 3-R_1	GCGTCTGTGATGGTATCAGC
exon 3-F_2	GCCAAGCAGACGAGGACTAC
exon 3-R_2	CCCGCCTTGGGCTACACAG
exon 4-F	CAGCCCTTTTTCTCCCGGGTTC
exon 4-R	CAGCCCTTCCAGACCCTGGG
exon 5-F	CAGAACTTGGGCCCCGTAGAC
exon 5-R	ACGTGACACACATTGGATGCAC
ASO[b]	
C−128	GGGATGGGAGGCGGGG
G−128	GGGATCGGAGGGGGGG
C405	CATCACAGACGCTGTGA
T405	TCACAGCATCTGTGATG
C433 (Q121)	TGCCCACCCAGGAACC
G433 (E121)	TGCCCACCGAGGAACC

[a] Primers used for the single-strand conformational polymorphism (SSCP) detection technique. F, forward; R, reverse.
[b] Primers used for allele-specific oligonucleotide (ASO) hybridization. Polymorphic alleles are indicated below.

For the C−19G and G230A (R53H) polymorphisms, we used the mutagenically separated PCR technique, in which normal and mutant alleles are amplified in the same tube with different-length, allele-specific primers (22). The following primers, in which additional differences (underlined) were introduced to correspond to the molecular variant and to reduce crossreactions between the two alleles, were designed: C−19G polymorphism: For−19C, 5'-GCAGGGCAGGGGTGGGGCTCTACGGGGATAAGGGCTTTTAAATGC-3'; For−19G, 5'-GAGGGGATAAGGGCTTTTAAAATG-3'; RevC−19G, 5'-GGCCCGTTCCC-CCTCCCAC-3'; R53H polymorphism: ForR53H, 5'-GCGTCTGT-GATGGTATCAGC-3'; Rev53R, 5'-TCCATTCCCATCTTTCCC-CAGACATTACCAGCTCTGGCTGGGACG-3'; Rev53H, 5'-CAATTACCAGCTCTGGCTGGGTGA-3'.

PCR were conducted in 25-μl reaction volumes containing 2.5 μl of 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3, 0.01% gelatin), 1.5 mM $MgCl_2$, 10 μM levels of each of the four dNTP, 10 pmol of each of the three primers, and 0.5 U of *Taq* polymerase. The first denaturation step at 94°C for 5 min was followed by 35 cycles of amplification at 94°C for 45 s, 58°C for 45 s, and 72°C for 45 s, with a final extension at 72°C for 7 min. The amplification reactions yielded 222- and 201-bp products for the R53 and H53 alleles and 187- and 166-bp products for the C−19 and G−19 alleles, respec-

tively. The multiallelic polymorphism in the promoter region between position −133 and position −121, described by Song *et al.* (12), was studied by direct sequencing.

Determination of Urinary Kallikrein Activity and Other Hormonal Parameters

Urinary kallikrein activity was assessed with a kinin-forming assay using bovine kininogen as a substrate (23), an esterase assay using ^{3}H-labeled *p*-tosyl-L-arginine-methyl ester (24), and an amidolytic assay using the chimeric, fluorogenic, peptide substrate D-PFF-NMec, which is highly specific for human kallikrein, is not cleaved by other kallikrein-related enzymes (25,26), and combines the carboxy-terminal sequence of bradykinin with a phenylalanine residue of the reactive loop of kallistatin (a specific kallikrein inhibitor) (27). Hydrolysis of D-PFF-NMec was measured with a Spectra Max Gemini microplate reader (Molecular Devices, Sunnyvale, CA), by incubating urine for 15 min at 37°C with 0.01 mM D-PFF-NMec in 200 μl of 20 mM Tris-HCl buffer (pH 9.0) containing 1 mM ethylenediaminetetraacetate.

Plasma renin activity was measured by RIA of angiotensin I, and active renin was quantified with an immunoradiometric assay (28). Plasma and urinary aldosterone levels were measured by RIA (29).

Production and Characterization of Recombinant Kallikrein Variants

The nonsynonymous SNP R53H and Q121E were studied via *in vitro* synthesis of recombinant kallikrein variants. Total mRNA from human kidney (Clontech, Palo Alto, CA) was reverse-transcribed and amplified by using the following two primers designed on the basis of the human renal prokallikrein cDNA sequence (10): forward primer, containing an added *Bam*HI restriction site, 5'-CGGGATCCTGGA-CACCTCTGTCACCATG-3'; reverse primer, with an added *Xba*I restriction site, 5'-GCTCTAGACAGGGCTGGGCGTTCAGGA-3'. The 823-bp prokallikrein cDNA obtained, beginning 19 bases before the initiator methionine codon and ending 15 bases after the stop codon, was subcloned into the expression vector pcDNA3 (Invitrogen, San Diego, CA). Both strands were sequenced, and no difference was observed between the sequence obtained here and that reported by Evans *et al.* (10), with codons for arginine at position 53 and glutamate at position 121.

Point mutations were separately introduced into the recombinant vector (TM site-directed mutagenesis kit; Clontech, Palo Alto, CA) by using the following oligonucleotides (the mutated codons are underlined): 5'-GGCTGGGTCACCACAACTTGTTTG-3' for the substitution of histidine for arginine at position 53 and 5'-AGTTGCCCACCGAGGAACCCGAAG-3' for the substitution of glutamate for glutamine at position 121. The mutations were confirmed by sequence analysis of the entire cDNA.

Expression of the kallikrein cDNA was performed via transfection of COS-7 cells (30). The transfected cells were cultivated for 24 h in medium containing 10% fetal calf serum and then for 48 h in serum-free medium. These cells secreted inactive prokallikrein, which was completely activated by incubation of the culture medium for 1 h at 37°C with 1 U trypsin immobilized on agarose beads (80 U/ml beads; Sigma Chemical Co., St. Louis, MO)/ml medium (31).

Urinary kallikrein was purified from a pool of human urine as described previously (31). The recombinant kallikreins were purified to homogeneity from serum-free COS-7 cell medium by a simplification of that procedure. After prokallikrein activation, the medium was dialyzed against 0.02 M phosphate buffer (pH 6.0) containing 0.05 M NaCl and was applied to a 1- × 10-cm diethylaminoethyl-Sephadex A-50 column (Pharmacia, Piscataway, NJ). Kallikreins were eluted with 0.02 M phosphate buffer (pH 6.0) containing 0.5 M NaCl, concentrated, and dialyzed against 0.05 M phosphate buffer (pH 8.2). The purified kallikreins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 12% gels at pH 6.8, under either nonreducing conditions (0.25 M Tris-HCl, pH 6.8, 6.9 mM sodium dodecyl sulfate) or reducing conditions (with β-mercaptoethanol).

The titration of urinary and recombinant kallikreins was performed by active site labeling with tritiated diisopropyl fluorophosphate ([^{3}H]DFP) (32), an irreversible serine protease inhibitor (specific activity, 8.4 Ci/mmol; NEN, Boston, MA). Unlabeled DFP was first assayed with urinary kallikrein, for determination of the experimental conditions for inhibition of kallikrein activity. The different kallikrein preparations were then incubated overnight at room temperature with various concentrations of [^{3}H]DFP, ranging from 2.7×10^{-6} to 10^{-5} M, in 0.6 ml of 0.2 M Tris-HCl buffer (pH 8.2). Unbound [^{3}H]DFP was eliminated by ultrafiltration with a Centricon filter device with a YM10 membrane (Millipore, Bedford, MA). The molarity of the kallikreins was determined by assuming stoichiometric incorporation of [^{3}H]DFP, as is the case for other serine proteases under similar conditions (32).

Enzymatic studies were performed with the D-PFF-NMec substrate described above and another intramolecularly quenched, fluorogenic substrate derived from the human kininogen sequence at the carboxy-terminal insertion site of bradykinin, o-aminobenzoic acid-FRSSRQ-ethylenediamine-2,4 dimitrophenyl (26). Substrate concentrations ranged from 5 to 78 × 10^{-6}M. The enzymatic activity was also studied with the kinin-forming assay (24), using increasing concentrations of bovine kininogen, ranging from 0.5 to 5 μg-lysine-bradykinin (Lys-BK) equivalents, incubated for 15 min at 37°C with the different enzymes. Enzfitter software (Elsevier Science Publishers, Amsterdam, The Netherlands) was used to fit the experimental data to the hyperbolic Michaelis-Menten rate equation. Values of k_{cat} were calculated with the equation $V_{max}/[E]_t = k_{cat}$.

The pH dependence of the kallikreins was investigated with the kinin-forming assay. Five microgram-Lys-BK equivalents of kininogen were incubated with each kallikrein in a citrate-phosphate-borate buffer (0.03 M citric acid, 0.03 M phosphoric acid, 17.4 M crystallized orthoboric acid, 1 N NaOH) with pH varying from 6.0 to 10.5, in increments of 0.5 units.

Statistical Analyses

Clinical and biologic characteristics are expressed as means and SD for continuous variables and as counts and percentages for discrete variables. The normality of each variable was assessed, and logarith-

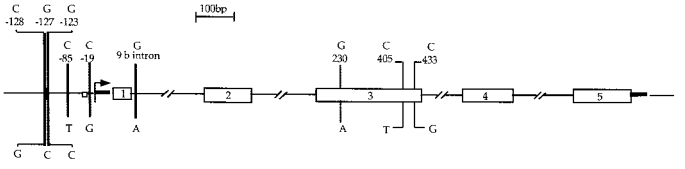

Figure 1. Schematic representation of the human kallikrein gene, indicating the locations of the polymorphisms. Numbered boxes indicate the exons. The TATA box located between −23 and −28 bp is represented by an open square followed by the transcription initiation site (arrow). The 5'- and 3'-untranslated regions are indicated by thick lines. Bases indicated above the diagram correspond to the sequence of the wild-type gene and those indicated below the diagram to the polymorphisms.

mic transformation was used when appropriate. The significance of urinary kallikrein excretion changes during alterations in sodium intake was determined by using the *t* test for paired values (Statview version 5.0 statistical software; Abacus Concepts Inc., Berkeley, CA). Univariate analyses were performed with generalized linear model regression techniques, and the results are expressed by using the Spearman correlation for quantitative variables and Fisher's exact test for qualitative variables (SAS version 6.12 statistical package; SAS Institute, Cary, NC). Multivariate analysis was performed by using the parameters that were demonstrated to be significantly related to urinary kallikrein excretion in the univariate analyses.

Comparison of the genotypic frequencies of SNP was performed by using contingency χ^2 tests. Pairwise-linkage disequilibrium was expressed as $D' = D/D_{max}$ or D/D_{min}, as described by Thompson *et al.* (33). The effect of each polymorphism was calculated with a simple ANOVA procedure.

Results

The locations of the nine SNP detected in the *hKLK1* gene are indicated in Figure 1 and Table 2. A complex single-strand conformational polymorphism pattern was observed for the region −170 to +1, upstream of the gene, corresponding to the five different SNP recognized by direct sequencing. A first group of three nucleotide substitutions were located in a 10-bp GC-rich sequence, *i.e.*, G→C at position −128, G→C at position −127, and G→C at position −123. Two other substitutions were a C→G transition at position −19 (2 bp downstream from a TATA box) and a C→T substitution at position −85. A G→A transversion was observed in intron 1, 9 bp downstream from the exon 1/intron boundary. One neutral and two missense SNP were observed in exon 3, *i.e.*, a C→T substitution at position 405 of the coding sequence (C405T), which conserves the aspartate residue at position 111 of the mature protein, a G→A transition at position 230, which leads to a change from arginine (CGC) to histidine (CAC) at position 53 (R53H), and a C→G transversion at position 433, which leads to a change from glutamine (CAG) to glutamate (GAG) at position 121 (Q121E).

Among the five SNP located in the 5'-part of the gene, only G−128C and C−19G were sufficiently frequent to be considered for further studies. The G→A transversion in intron 1 was observed for a single subject. Two of the polymorphisms detected in exon 3, namely R53H and C405T, were significantly more common among the Afro-Caribbean subjects (Table 2). Most of the polymorphisms were in complete linkage disequilibrium with each other (Table 3). Despite differences in allelic frequencies, similar patterns of disequilibrium were observed for the two ethnic groups (data not shown). The R53H and Q121E mutations did not exhibit significant linkage disequilibrium, but the C−19G polymorphism did exhibit strong linkage disequilibrium with R53H and occurred with almost the same frequency. The R53H polymorphism was also in linkage disequilibrium with the G−128C polymorphism, which was located in a multiallelic promoter region with 10 identified variations (12). Further analysis of allelic distribution among 78 R53R and 75 R53H Caucasian subjects indicated that the R53H mutation was in complete linkage disequilibrium with one of these alleles, the I allele [according to the nomenclature used by Song *et al.* (12)] (data not shown).

Allelic frequencies for the five most frequent SNP were studied in the two hypertensive populations and the normotensive control group (Table 4). All genotypic frequencies satisfied the Hardy-Weinberg equilibrium. No significant association was observed between any of these SNP and hypertension. The two hypertensive populations were biologically characterized, and one was studied with a contrasted salt regimen. As expected, marked increases in plasma renin activity, active renin concentrations, and plasma and urinary aldosterone levels were observed with the low-sodium diet, together with a significant increase in urinary kallikrein excretion (Table 5). Systolic or diastolic BP was not correlated with urinary kallikrein activity with either diet.

When urinary kallikrein activity levels were analyzed on the basis of the *hKLK1* polymorphisms, no relationship was observed with either the polymorphisms located in the promoter region (G−128C and C−19G) or the Q121E missense mutation (data not shown). Conversely, a significant association with the R53H polymorphism was observed for both groups of

Table 2. Allelic frequency of polymorphisms in the *hKLK1* gene

Variant	Location	Position	Substitution		Allelic Frequency	
			Nucleotide	Amino Acid	Caucasian (n = 46)	African (n = 40)
1	5′	−128	G→C		0.29	0.31
2	5′	−127	G→C		0.02	
3	5′	−123	G→C			0.01
4	5′	−85	C→T		0.03	
5	5′	−19	C→G		0.04	0.14
6	Intron 1	Intron 1	G→A		0.01	
7	Exon 3	+230	G→A	R→H (53)	0.03	0.07[a]
8	Exon 3	+405	C→T	D→D (111)	0.35	0.17[a]
9	Exon 3	+433	C→G	Q→E (121)	0.33	0.35

[a] $P < 0.05$, compared with Caucasian subjects.

Table 3. Linkage disequilibrium between polymorphisms of the *hKLK1* gene

	D'[a]			
	C−19G	G230A (R53H)	C405T	C433G (Q121E)
G−128C	−1	−1	−0.693[b]	−0.519
C−19G		1	−1	−1
G230A (R53H)			−1	0.452[b]
C405T				0.835

[a] Calculations were performed by using contingency χ^2 tests. Pairwise linkage was expressed as $D' = D/D_{max}$ (33). D' values of 1 or −1 depend on the disequilibrium with either the wild-type or mutated allele. Values close to 0 indicate the absence of disequilibrium between two polymorphisms. All values in this table are highly significant, except as indicated.
[b] Not significant.

Table 4. Clinical characteristics of the hypertensive and normotensive subjects and allelic frequencies[a]

	Control Subjects (n = 255)	Hypertensive Subjects	
		Population 1 (n = 74)	Population 2 (n = 240)
Clinical characteristics			
age (yr)	43.8 ± 6.7	47.1 ± 8.9	48.3 ± 7.5
gender (% male)	41	41	49
BMI (kg/m^2)	23.5	25.0 ± 2.5	27.5 ± 3.0
SBP (mmHg)	113.3 ± 9.2	162 ± 16	157 ± 21
DBP (mmHg)	71.5 ± 6.5	105 ± 7	98 ± 11
Allelic frequencies			
C−128G	0.27	0.32	0.27
C−19G	0.03	0.03	0.04
G230A (R53H)	0.04	0.03	0.03
C405T	0.29	0.33	0.31
C433G (Q121E)	0.49	0.45	0.24

[a] BMI, body mass index; SBP, systolic BP; DBP, diastolic BP.

subjects (Table 5). In the first group, with an *ad libitum* salt diet, urinary kallikrein activity noted for the heterozygous R53H subjects was approximately 50% of that observed for the wild-type subjects (Table 5). In the second group, for which kallikrein was measured with the esterase assay, urinary kallikrein activity was only slightly lower for the R53H subjects with the high-salt diet; however, the difference between genotypes was accentuated and became significant with the low-salt diet, which stimulated kallikrein synthesis. Urinary kallikrein activity was also measured for 66 individuals in this group with the specific kallikrein substrate D-PFF-NMec. The genotype effect was confirmed (Table 5). Because multiple factors can affect urinary kallikrein activity, univariate and multivariate analyses were conducted for both groups. For the first population (n = 74), the only significant correlate was the urinary aldosterone level (r = 0.43, $P < 0.001$). Differences in urinary kallikrein activity according to genotype were still significant after adjustment for this parameter (P = 0.03). For the second population (n = 164), the urinary aldosterone level ($P < 0.01$) was also the main variable related to the trait with the high-sodium diet. In addition to the urinary aldosterone level, plasma renin activity (r = 0.45, $P < 0.01$) and urinary potassium excretion (r = 0.28, P = 0.02) were significantly related to urinary kallikrein activity with the low-sodium diet. In the multivariate regression analysis, the R53H genotype was associated with urinary kallikrein activity with both diets (P = 0.04 and P = 0.06, respectively).

The concentrations of the purified recombinant kallikrein preparations, as determined by active site titration, ranged from 4 to 32 nM. The recombinant kallikrein variants and urinary kallikrein displayed behavior typical of tissue kallikrein in polyacrylamide gel electrophoresis, with a greater apparent molecular mass under reducing conditions, compared with nonreducing conditions. The electrophoretic migration patterns of all of the enzymes were identical, suggesting that glycosylation levels were similar for urinary and recombinant renal kallikreins (data not shown).

The Q121E mutation slightly decreased the catalytic constants for hydrolysis of the kininogen-derived fluorogenic substrates, but the kinetic parameters remained of the same order of magnitude as those obtained for urinary and wild-type recombinant kallikreins (Table 6). In contrast, the 53H kal-

Table 5. Clinical and hormonal characteristics of hypertensive patients, according to the R53H polymorphism[a]

	Population 1, *Ad Libitum* Diet		Population 2			
			High-Na^+ Diet		Low-Na^+ Diet	
	RR (n = 68)	RH (n = 6)	RR (n = 151)	RH (n = 13)	RR (n = 151)	RH (n = 13)
Age (yr)	47.3 ± 8.8	44.8 ± 9.5	48.4 ± 7.4	46.8 ± 7.4		
Gender (M/F)	46/22	5/1	82/69	6/7		
BMI (kg/m^2)	25.0 ± 2.5	25.3 ± 2.4	26.6 ± 13.1	28.0 ± 5.0		
SBP (mmHg)	163.7 ± 13.1	165.3 ± 13.7	150.5 ± 19.3	155.0 ± 19.5	137.3 ± 29.0	135.4 ± 14.2
DBP (mmHg)	105.6 ± 6.8	103.7 ± 6.4	88.9 ± 12.3	90.2 ± 10.0	81.6 ± 11.9	80.9 ± 7.4
Plasma						
active renin (pg/ml)			14.8 ± 12.0	10.5 ± 5.9	36.1 ± 29.0	27.6 ± 14.0
PRA (ng/Ang I per ml)	0.8 ± 0.7	0.6 ± 0.5	0.69 ± 0.59	0.53 ± 0.59	2.3 ± 2.1	1.9 ± 1.7
aldosterone (μg/24 h)	116 ± 54	154 ± 102	68.0 ± 46.2	56.7 ± 4.7	182 ± 107	199 ± 15
Urine						
Na^+ (mmol/24 h)	118 ± 60	132 ± 45	205 ± 70	199 ± 67	15.0 ± 9	16.0 ± 8
K^+ (mmol/24 h)	65 ± 35	68 ± 42	67 ± 20	66 ± 26	71 ± 19	66 ± 18
aldosterone (μg/24 h)	8.3 ± 4.9	9.4 ± 3.4	13.7 ± 8.2	15.6 ± 7.7	30.9 ± 21.1	36.0 ± 19
Urinary kallikrein						
kinin-forming assay (pg LBK/min per 24 h)	106 ± 82	58 ± 59[b]				
esterase assay (TU/24 h)			0.519 ± 0.394	0.400 ± 0.282	1.021 ± 0.813	0.511 ± 0.464[c]
D-PFF-NMec assay (ΔFU/24 h)			15.9 ± 2.05[d]	1.53 ± 0.82[c,e]	36.3 ± 3.71[d]	9.12 ± 2.36[c,e]

[a] The low-Na^+ diet had a significant effect ($P < 0.05$) on the active renin concentration, plasma renin activity (PRA), plasma and urinary aldosterone concentrations, and urinary kallikrein activity (not shown). BMI, body mass index; SBP, systolic BP; DBP, diastolic BP; LBK, lysine-bradykinin; Ang I, angiotensin I; TU, TAME unit, (millimole p-tosyl-L-arginine methyl ester hydrolyzed/mn; FU, fluorescence unit; Nmec, 7 amino 4 methyl coumarin; RR, R53 homozygotes; RH, R534 homozygotes.
[b] $P = 0.08$.
[c] $P < 0.05$, RH compared with RR.
[d] $n = 61$.
[e] $n = 5$.

likrein variant displayed a 10-fold increase in K_m values for both substrates and a much lower k_{cat} value for Abz-FRSSRQ-EDDnp. The kinin release capacities of the urinary, recombinant wild-type, and Q121E kallikreins were also similar (Table 6). The R53H enzyme exhibited very low activity, and Lys-BK was detected only with an extended incubation time of 2 h. The amounts of Lys-BK released by using 5 μg-Lys-BK equivalents of kininogen and 0.05 nM enzyme were 2.13 ng/ml per min for urinary kallikrein, 0.68 ng/ml per min for recombinant wild-type kallikrein, 0.74 ng/ml per min for Q121E kallikrein, and 0.006 ng/ml per min for R53H kallikrein. It was therefore not possible to calculate kinetic parameters for the hydrolysis of kininogen by R53H kallikrein.

The mutations did not significantly affect the pH dependence of the enzyme activity. Optimal activity of urinary and recombinant kallikreins was obtained at pH values between 8.0 and 9.5. The enzymes all exhibited only minor activity differences between pH 6.0 and pH 10.0, and activity at pH 7.0 was ≥70% of the optimal pH activity in all cases (data not shown).

Discussion

This study identifies several molecular variants of the kallikrein gene and demonstrates that one missense polymorphism that is present in 6 to 8% of Caucasian subjects has a major effect on enzyme activity and is associated with a decrease in urinary kallikrein activity. This study documents a genetic polymorphism of kallikrein activity and its molecular basis.

The polymorphisms detected in this study include those described by Berge *et al.* (14) at positions −127 and 230 (R53H) and by Song *et al.* (12) at positions −127, −128, and −123 in the promoter region. The C433G (Q121E) mutation had not been previously described as a polymorphism, but Chan *et al.* (34) reported this sequence variation in a comparison of cDNA from various human organs. Our findings sug-

Table 6. Kinetic parameters for human urinary kallikrein (hK1) and wild-type and mutated (R53H and Q121E) recombinant kallikreins

	Abz-FRSSRQ-EDDnp[a]			D-PFF-NMec[b]			Bovine Kininogen		
	K_m (μM)	k_{cat} (min^{-1})	$\frac{k_{cat}}{K_m}$ ($\mu M^{-1} \cdot min^{-1}$)	K_m (μM)	k_{cat} (min^{-1})	$\frac{k_{cat}}{K_m}$ ($\mu M^{-1} \cdot min^{-1}$)	K_m (μM)	k_{cat} (min^{-1})	$\frac{k_{cat}}{K_m}$ ($\mu M^{-1} \cdot min^{-1}$)
hK1	3.66 ± 1.17	9.04 ± 0.96	2.47	7.27 ± 1.80	83.7 ± 7.2	11.5	1.71 ± 0.72	56.8 ± 15.8	33.2
Wild-type	1.83 ± 0.5	11.0 ± 0.9	6.01	7.37 ± 3.50	30.7 ± 3.8	4.2	7.86 ± 4.00	76.0 ± 34.7	9.7
Q121E	1.91 ± 0.36	2.25 ± 0.1	1.18	6.69 ± 3.75	14.0 ± 2.0	2.1	1.93 ± 0.44	62.4 ± 9.6	32.3
R53H	43.6 ± 9.5	0.09 ± 0.01	0.002	89.2 ± 23.7	11.3 ± 0.2	0.13	c	c	c

[a] Fluorogenic substrate mimicking the carboxy-terminal cleavage site of kininogen (o-aminobenzoic acid-FRSSRQ-ethylenediamine 2, 4 dinitrophenyl).
[b] Fluorogenic chimeric amidated substrate derived from the carboxy-terminal sequence of bradykinin and the kallistatin loop (D-PFF-7 amino 4 methyl conmarin).
[c] Kininogen hydrolysis was too slow to allow determination of kinetic parameters.

gest that that apparent interorgan variation was probably related to the transcription of polymorphic alleles, a hypothesis consistent with the presence of a single gene (the *hKLK1* gene) coding for tissue kallikrein in the human genome and the high frequency of the Q121E polymorphism. The polymorphisms at positions −85, −19, and 405 and intron 1 were also not reported previously. DNA analysis of our groups of subjects did not reveal the polymorphism in the fourth exon reported by Evans *et al.* (10), which probably occurs with low frequency. This study increases the number of possible SNP at the *hKLK1* locus, with nine identified nucleotide substitutions, of which five [at positions −128, −19, 230 (R53H), 405, and 433 (Q121E)] can be considered to be sufficiently frequent to be tested in future studies.

The R53H and Q121E polymorphisms were located in exon 3, coding for the active site (10), and both resulted in partial charge modification. The R53H polymorphism was associated with reduced urinary kallikrein activity. The functional consequences of these amino acid mutations were therefore assessed by studying purified, titrated, recombinant variants. The mutation of Arg53 to histidine led to a major decrease in the catalytic efficiency in cleaving kininogen-derived peptide substrates and in releasing kinins from kininogen. Results obtained with the Abz-FRSSRQ-EDDnp substrate, which mimics the kininogen sequence around the carboxy-terminal cleavage site for the release of Lys-BK, suggest that the 53H variant cleaves kininogen inefficiently at this location.

Kallikreins have an extended substrate binding site, involving substrate residues located on both sides of the scissile bond and far away from it (35,36). On the basis of structural data obtained from the crystallographic analysis of human kallikrein (37,38) and molecular modeling studies of the interaction of rat kallikrein with a tetrapeptide inhibitor (35), Arg53 would be located in the prime side of the substrate binding site of the protease, *i.e.*, the region of the active site that interacts with amino acids carboxy-terminal to the scissile bond in substrates (Figure 2). The major changes in the kinetic constants K_m and k_{cat} indicate that Arg53 influences both the binding and cleavage of substrates. This is more apparent for Abz-FRSSRQ-EDDnp than for D-PFF-NMec, possibly because the former,

Figure 2. Ribbon representations of the three-dimensional structure of human tissue kallikrein, demonstrating the locations of amino acids at position 53 and 121. Residues of the catalytic triad of His41, Asp96, and Ser189 in the active site pocket, together with Arg53 and Gln121, are shown with their full structures. This figure was prepared with the program Molmol (38) and rendered with Povray 3.0 (www.povray.org).

which is cleaved within its peptide moiety at the arginine-serine bond, has several residues in the prime position, whereas the latter, which is cleaved after the two phenylalanines, has none. These results are in agreement with the observation that the proteolysis of kininogen and the release of kinins are strongly affected by Arg53. Arg53 is probably involved in this function via its positively charged side chain. This is further suggested by the conservation of this amino acid among mammalian species except for mice and the fact that it has been replaced in mice by another positively charged residue, *i.e.*, lysine (39,40).

In contrast, the amino acid at position 121 is not in the vicinity of the active site and is therefore unlikely to participate in substrate binding and catalysis (Figure 2). Accordingly, little difference in enzyme activity was observed for kallikrein variants with a negatively charged glutamate residue *versus* a noncharged polar glutamine residue at position 121 (Table 6) (34). This is consistent with the observation that, unlike Arg53, the amino acid charge at position 121 is not conserved among mammalian species; either a glutamate residue (39,40) or a glutamine residue (10) can be observed at this location. No association was observed between the Q121E polymorphism and urinary kallikrein activity, further suggesting that this polymorphism has no physiologic consequences.

The R53H polymorphism, however, is associated with the level of urinary kallikrein activity. Only heterozygotes could be studied, because of the low prevalence of the polymorphism. These subjects exhibited, on average, one-half the urinary kallikrein activity level of the R53 homozygotes. This finding was consistent in the two populations studied, under conditions of *ad libitum* sodium intake and after stimulation of kallikrein synthesis with low sodium intake. The fact that, with the *p*-tosyl-L-arginine-methyl ester esterase assay, the genotype effect seemed less marked with the high-sodium diet, compared with the low-sodium diet, can be explained by the presence of other esterases in urine (41). These *in vivo* observations are consistent with the loss of enzyme activity induced by the mutation. However, because the R53H mutation is in strong linkage disequilibrium with other polymorphisms located in the promoter region, it may also be associated with alterations in gene transcription. However, one promoter allelic form that is in complete linkage disequilibrium with the R53H mutation, *i.e.*, the I allele, has been demonstrated to exhibit no altered transcriptional activity *in vitro* (12). The development of variant-specific immunologic assays could facilitate investigation of this issue.

The observation of a loss-of-function polymorphism in the kallikrein gene may lead to speculation regarding the physiologic consequences of constitutively low kallikrein activity in the kidney. The first trait that may be affected is the regulation of BP. The results obtained in our study do not support an association between the R53H polymorphism and BP. The same observation was made by Berge *et al.* (14) among normotensive subjects. This absence of association needs to be confirmed in larger studies. The effects of kallikrein polymorphisms on BP may be more subtle and may depend on the environment, especially sodium and potassium levels in the diet, which affect kallikrein synthesis (Table 5) (7). In this respect, it is interesting to note that low urinary kallikrein activity has been associated with salt sensitivity of BP and with increased sensitivity to thiazide diuretics (1,42). Reduced kallikrein activity may alter the regulation of renal blood flow (1,2). Kallikrein is also synthesized in arteries, and mice engineered for inactivation of the kallikrein gene exhibit reduced arterial vasodilatory capacity (43). Studies of 53H kallikrein carriers with respect to renal and cardiovascular regulation are thus warranted. Analysis of this polymorphism among patients with renal or vascular diseases may help establish the role of the kallikrein-kinin system in the progression of these diseases.

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