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α1 Na,K-ATPase and Na,K,2Cl-Cotransporter/*D3mit3* Loci Interact to Increase Susceptibility to Salt-Sensitive Hypertension in Dahl S^{HSD} Rats

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Abstract

Background: Essential (multigenic) hypertension is a complex multifactorial disease whose genetic etiology has not been unraveled on a major locus-effect investigative paradigm. As with other complex genetic diseases, applying an interacting loci paradigm could be critical in the elucidation of genetic determinants. Having defined the α1 Na,K-ATPase (α1NK) as a hypertension susceptibility gene in Dahl salt-sensitive (Dahl S) rats, we determined whether α1NK interacts with another renal epithelial Na transporter to increase susceptibility to salt-sensitive hypertension. We focused on α1NK and Na,K,2Cl-cotransporter (NKC) as an a priori candidate interacting gene pair because they comprise a functionally linked Na transport system in renal thick ascending limb of Henle (TALH) epithelial cells and exhibit altered function in prehypertensive Dahl S rats in contrast to Dahl salt-resistant normotensive (Dahl R) rats.

Material and Method: Cosegregation analysis of α1NK and NKC loci was done in a (Dahl S × Dahl R) F2 cohort characterized for blood pressure by radiotelemetry using the *D2mgh11* microsatellite marker in the α1NK gene and the *D3mit3* microsatellite marker close to the NKC gene (NKC/*D3mit3* locus). Single locus and digenic analyses were performed to establish the individual and interactive genetic contribution to salt-sensitive hypertension. Molecular analysis was then done to support the NKC gene as the likely candidate gene interacting with α1NK in Dahl salt-sensitive hypertension pathogenesis.

Results: Compared with respective single locus analysis, digenic analysis of 96 F2 (Dahl S × Dahl R) hybrid male rats revealed cosegregation of α1NK and NKC/*D3mit3* loci as interacting pair with salt-sensitive hypertension with markedly increased significance for systolic (one-way ANOVA $p = 10^{-6}$), diastolic ($p = 10^{-5}$), and mean arterial ($p = 10^{-6}$) blood pressures. Concordantly, two-way ANOVA detected interaction between α1NK and NKC loci in determining the levels of systolic ($p = 0.004$), diastolic ($p = 0.008$), and mean arterial ($p = 0.006$) pressures. To unravel potential NKC molecular dysfunction(s) involved in hypertension pathogenesis, we investigated putative differences between Dahl S and Dahl R rats in nucleotide sequence and isoform gene expression of the renal-specific Na,K,2Cl-cotransporter. Molecular analysis revealed an inversion of alternatively spliced NKC-isoform ratios (4B:4A:4F) between Dahl S and Dahl R prehypertensive kidneys supported by four mutations in intron-3 immediately upstream to alternatively spliced exons 4B, 4A, and 4F. No nucleotide changes were detected within the aminoacid encoding exons of NKC.

Conclusions: Altogether, these current data and previous characterization of the role of the Q276L α1NK molecular variant in Dahl S hypertension provide cumulative compelling evidence that α1NK and NKC/*D3mit3* loci interact to increase susceptibility to hypertension in Dahl S rats and that NKC is the likely candidate gene that interacts with α 1NK. More importantly, the data substantiate gene interaction as an operative mechanism in multigenic hypertension.

Introduction

Spontaneous abnormal sodium handling is a distinct pathophysiologic characteristic exhibited in salt-sensitive hypertension as modeled by the Dahl salt-sensitive hypertensive rat (1–3) prompting the hypothesis that genes involved in sodium homeostasis are putative candidate genes for salt-sensitive hypertension susceptibility. We have recently identified the α1 Na,K-ATPase (α1NK) gene as a hypertension susceptibility gene in the Dahl S rat model (4). This enzyme is the sole active sodium transporter in the basolateral membrane of all renal tubular epithelial cells (5,6). In the apical membrane, a number of different transporters are involved in sodium handling throughout the nephron (5). Among them, the renal-specific bumetanide-sensitive Na,K,2Cl-cotransporter (NKC) is a key transport system in the thick ascending

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limb of the loop of Henle (TALH) (5). Three isoforms of the renal Na,K,2Cl-cotransporter (NKC-B, NKC-A, and NKC-F) produced by the differential splicing of three mutually exclusive alternative splicing exons (exons 4B, 4A, and 4F) have been characterized (7–10). These isoforms exhibit distinct patterns of expression throughout the rat nephron (9) and are presumed to have distinct transport kinetic properties (7). The NKC-F isoform is detected exclusively in the medullary TALH and the outer medullary collecting duct (9); the NKC-A isoform in cortical and medullary TALH, as well as in macula densa-containing segments (9); and isoform NKC-B primarily in cortical TALH and macula densa-containing segments (9). Several lines of evidence have implicated the NKC system in salt-sensitive hypertension (11–13). The Dahl S rat has been found to have an altered pressure natriuresis and increased Cl^- reabsorption in TALH compared with Dahl R rats (11–13). The pressure-natriuretic relationship was found to be blunted in Dahl S rats prior to the development of hypertension when compared with Dahl R rats (11), and was associated with direct elevation of Cl^- transport in TALH (12,13). These data implicate a potential role of renal-specific NKC in hypertension pathogenesis. Because the α1 Na,K-ATPase gene was previously determined to be a hypertension susceptibility gene in the Dahl S rat model (4), potential interaction of the NKC locus with the α1 Na,K-ATPase locus (marked by *D2mgh11*) becomes a logical hypothesis based on a biological basis for gene interaction. Functional interaction between the α1 Na,K-ATPase and the renal-specific NKC is supported by the following: a) both share substrates because the identical ions are transported (Na^+, K^+); b) both colocalize to the identical renal epithelial cell, with the α1 Na,K-ATPase in the basolateral membrane and the renal-specific NKC in the apical membrane of the thick ascending limb; and c) their respective functions are interdependently linked; that is, the Na^+ gradient established by the α1 Na,K-ATPase provides the downhill energy for the apical NKC while the entry of Na^+ ions activates α1 Na,K-ATPase. It is therefore conceivable that the presence of a mutant α1 Na,K-ATPase and a dysfunctional NKC in TALH epithelial cells could result in multiplicative dysfunction of sodium transport leading to increased susceptibility to salt-sensitive hypertension.

To test this hypothesis, we performed two modes of analysis to investigate putative gene interaction. The results reported here demonstrate that α1NK and NKC (marked by *D3mit3*) loci interactively increase susceptibility to salt-sensitive hypertension in Dahl S rats, thus providing a genetic framework readily testable in different human essential hypertensive populations.

Materials and Methods

Cosegregation Analysis

The establishment of the (Dahl S male × Dahl R female) F2 male cohort and phenotypic characterization has been described previously (4). Briefly, nonstressed 24-hr average blood pressure measurements were obtained by radiotelemetry (4). After 8 weeks of high salt (8% NaCl) challenge, the F2 hybrid rats were sacrificed and tail genomic DNAs isolated. Genotyping was performed using the following microsatellite markers: *D2mgh11* (α1NK), *D3rat59, D3rat6, D3rat10, D3mit3, D3rat18, D3rat26, D3rat44,* and *D3rat100* obtained from Research Genetics (Huntsville, Ala).

Cloning and Nucleotide Sequencing of Dahl S and Dahl R NKC cDNAs

Using a probe spanning nt 32–726 (derived by RtTh-PCR of rat kidney RNA) of the reported Sprague-Dawley rat renal-specific NKC cDNA (14) (a region chosen because it displays the least homology with the Na,Cl-cotransporter), cDNAs from Dahl S and Dahl R rat kidney cDNA libraries were isolated. A full-length Dahl S cDNA and several partial-length Dahl R cDNAs were isolated and sequenced. The nucleotide sequence spanning the entire amino acid coding region for both Dahl S and Dahl R cDNAs were obtained by the dideoxy chain termination method using the Sequenase Sequencing Kit (USB, Cleveland, OH) as described previously (15,16). Appropriate oligonucleotide primers utilized in the sequencing reactions were obtained from Research Genetics, based on the reported Sprague-Dawley NKC sequence (14).

Sequence Analysis of NKC Genomic Fragments

A 1.45-kb genomic DNA fragment containing the B, A, and F alternative spliced exons was amplified by polymerase chain reaction (PCR) from Dahl S and Dahl R rat genomic DNAs using an upstream intron 3-specific primer (B2.1: 5′-TTC-AAC-AAC-TAC-GTA-GGT-GTG-3′) localized 5′ to cassette B and a downstream intron 6-specific primer (F1.1: 5′-TTC-CAA-GTC-CAG-TTA-GCA-ACA-3′) localized 3′ to cassette F. Both Dahl S and Dahl R 1.45-kb fragments were recovered from a 0.9% low-melting-point agarose gel and completely sequenced directly by cycle-sequencing using the Perkin Elmer Branchburg, NJ, cycle-sequencing kit as described previously (16). Sequencing primers were B2.1; Ar (5′-GGA-GCA-TGT-GCC-GCG-GAT-ATT-3′), an intron 5-specific primer localized 3′ to cassette A; and F1.1. Similarly, a 1.3-kb genomic DNA fragment containing intron 2 (upstream primer 5′-TGT-TCA-TTG-ACC-AAC-TAC- TGT-G-3′ within exon 2; downstream primer 5′-GTG- ACC-TTC-ATC-TCA-CAT- TCA-G-3′ within exon 3) and a 4.6-kb genomic DNA fragment containing intron 3 (upstream primer 5′-CTC-TCC-TGG-ATT-GTA-GGA-GAA-G-3′ within exon 3; downstream primer 5′-TGT-CAC-GGC-TAA-GCC-GAT-G-3′ within exon 4B) were PCR-amplified from Dahl S and Dahl R genomic DNAs, subcloned into Psp73 plasmid vector and subsequently sequenced. Both DNA strands were sequenced to corroborate nucleotide sequences obtained.

Analysis of NKC mRNA Levels

Quantitation of total NKC mRNA levels and isoform NKC-A–, NKC-B–, and NKC-F–specific mRNA levels was done by densitometric analysis of reverse transcriptase polymerase transcription reaction (RT-PCR) products derived from total kidney RNA isolated from prehypertensive Dahl S ($n = 6$) and Dahl R ($n = 6$) rats at 10 weeks of age on regular rat chow. RT-PCR products were size fractionated by 2% agarose gel electrophoresis. The ethidium bromide–stained fragments were photographed and quantified by image densitometric analysis. RT-PCR conditions for each isoform were optimized in terms of number of cycles and amount of total RNA used to perform the quantitation in the linear range of PCR product formation for each isoform, as well as for the assessment of total NKC mRNA. Thus, the following specific conditions were applied: all PCR reactions (20 μL reaction volume) were preceded by a single cDNA synthesis step (50°C × 30 min) followed by 30 cycles with 1.0 μg total RNA for Dahl S and Dahl R NKC-B; 0.05 μg total RNA for Dahl S NKC-A; 0.2 μg total RNA for Dahl R NKC-A; 0.4 μg total RNA for Dahl S NKC-F; 0.2 μg total RNA for Dahl R NKC-F, and 0.1 μg total RNA for Dahl S and Dahl R NKC-total and β-actin. Each cycle consisted of 94°C × 30 sec (denaturation), 54°C × 30 sec (annealing), and 70°C × 30 sec (elongation) using the GibcoBRL (Rockville, MD) RT-PCR kit components. The following primers were utilized for the RT-PCR analysis: for NKC-B, upstream primer 5′-GAG-CAA-GCA-GAA-AAC-AAG-G-3′, downstream primer 5′-TTG-TGG-CGA-TAG-CAG-AGG-3′ (223 bp product); for NKC-A, upstream primer 5′-GAG-CAA-GCA-GAA-AAC-AAG-G-3′, downstream primer 5′-TGA-CAA-CCC-AGT-GAT-AGA-GG-3′ (203 bp product); for NKC-F, upstream primer 5′-GAG-CAA-GCA-GAA-AAC-AAG-G-3′, downstream primer 5′-ACC-TGT-GAG-TGT-TGT-CAC-TAC-C-3′ (196-bp product); for NKC-total, upstream primer 5′-AAA-CAG-GGA-AAA-GGC-ACG-3′, downstream primer 5′-TCT-TTT-CCT-TGA-CTG-CTT-CC-3′ (384 bp product); for β-actin, upstream and downstream primers (289-bp product) were obtained from Research Genetics. β-Actin was utilized as control. Quantitative data are shown as percent of each isoform detected within the total NKC mRNA sequences present in the kidney.

Statistical Analysis

For single locus and digenic interaction analysis, blood pressure parameters and genotypes were analyzed by one-way analysis of variance (one-way ANOVA; SIGMA STAT, Jandel Scientific, San Rafael, CA). Gene interaction analysis was also assessed by two-factor ANOVA (two-way ANOVA; SIGMA STAT, Jandal Scientific, San Rafael, CA). Blood pressure is assigned as the variable under consideration, and the two factors being simultaneously tested for interaction are α1NK genotype (first factor) and NKC genotype (second factor).

Results

Single Locus Analysis

To assess the genetic contribution of the NKC locus (marked by the *D3mit3* marker, NKC/*D3mit3*) to salt-sensitive hypertension susceptibility, we performed cosegregation analysis on 96 F2 (Dahl S male × Dahl R female) hybrid male rats characterized phenotypically for systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), heart rate, and activity by radiotelemetry after 8 weeks of high-salt (8% NaCl) diet challenge. Database search of Gene Bank + EMBL + DDBJ + PDB sequences deposited in the NCBI library and human/mouse/rat homology region maps (17) localized the rat NKC gene to rat chromosome 3, about 3 cM from the rat *B2M* locus, placing it in close proximity to *D3mit3* marker (Fig. 1). Chromosome 3 scan was then done with eight markers informative for our Dahl S × Dahl R intercross. As shown in Fig. 1 and Table 1, one-way ANOVA p values peaked at the *D3mit3* marker for SBP, DBP, and MAP. Because NKC is the only candidate gene with a documented patho-physiologic basis (12,13) closest to *D3mit3,* the *D3mit3* region is hereafter referred to as the NKC/ *D3mit3* locus. Statistical significance at the NKC/ *D3mit3* locus (SBP, $p = 0.036$; DBP, $p = 0.02$; MAP, $p = 0.023$; Table 1) and α1NK locus (SBP, $p = 0.006$; DBP, $p = 0.015$; MAP, $p = 0.011$; Table 2) meet the threshold criterion ($p < 0.05$) for first level preliminary detection of putative interacting loci in a two-step digenic analysis as was done for susceptibility to diabetes (18). We note, however, that although p values for α1NK locus fulfill the p value threshold ($p < 0.01$) for confirmed linkage having been previously demonstrated in a Dahl S × Dahl R F2 cohort (4), p values for the NKC locus do not attain levels indicative of suggestive linkage (19) as a single gene locus. We also note that the NKC locus on chromosome 3 reported here is distinct from two previously characterized chromosome 3 blood pressure quantitative trait loci (QTLs) (20,21). More specifically, the NKC locus (*D3mit3*) is 16 cM away from the endothelin 3 (*Edn 3*) gene blood pressure QTL (Fig. 1) detected in a male F2 (Dahl S × Brown Norway) cohort (20) and 36 cM away from a second blood pressure QTL detected near *D3mgh6* (Fig. 1) in a male F2 (Dahl S × Lewis) cohort (21). Conversely, chromosomal regions flanking the *Edn 3* QTL and *D3mgh6* QTL did not cosegregate with salt-sensitive hypertension in our male F2 (Dahl S × Dahl R) cohort (Fig. 1). Differences in cosegregation results presented here and in previous studies (20,21) are most likely due to different normotensive strains used in the F2 intercross as suggested previously (22), as

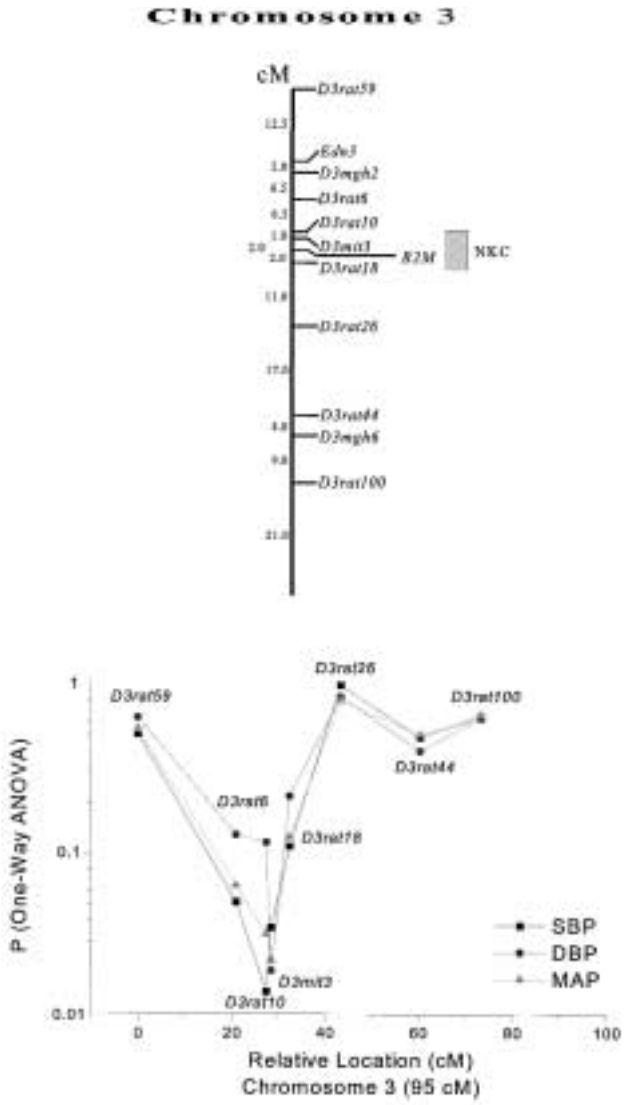

Fig. 1. Cosegregation analysis of Na,K,2Cl-cotransporter (NKC) locus with salt-sensitive hypertension in F2(Dahl S × Dahl R) hybrid male rats. Total chromosome 3 scan analyzing marker cosegregation with SBP, DBP, and MAP measured after 8 weeks of a high-salt (8% NaCl) diet. Markers informative for the Dahl S and Dahl R hybrid cross are shown at the top and are marked along their respective relative locations on chromosome 3 in centimorgans (cM). Parallel correlation trends along chromosome 3 are observed for SBP, DBP, and MAP peaking at *D3rat10* and *D3mit3* in proximity to *β2M,* which is about 3 cM away from the NKC locus.

well as due to differential phenotypic characterization of cohorts, diet challenge (type of salt and duration), and blood pressure determination [tail cuff method (20,21) versus radiotelemetry in this report].

Gene Interaction Analysis

Two modes of analysis were applied to investigate putative gene interaction between α1NK and NKC loci. First, having fulfilled the threshold criterion ($p < 0.05$) to validate digenic analysis, one-way ANOVA was performed to determine α1NK–NKC loci interaction. We determined whether homozygosity for the S alleles at both loci ($[S^{NK}S^{NK} + S^{NKC}S^{NKC}]$) carried increased risk for susceptibility to hypertension compared with rat groups with genotypes carrying two or more R alleles and homozygous in at least one locus ($[R^{NK}R^{NK} + X^{NKC}X^{NKC}]$ or $[X^{NK}X^{NK} + R^{NKC}R^{NKC}]$ where X = S or R). This a priori stratification was designed based on the observation in F1 (Dahl S × Dahl R) male rats that average blood pressure levels (SBP 152 ± 1.4 mmHg) were closer to Dahl R male rat group average (SBP 130 ± 1.3 mmHg) than to Dahl S male rat group average (SBP 220 ± 7.1 mmHg) (4), thus suggesting "overall" recessiveness of the cumulative polygenic hypertension phenotype. This observation leads to the a priori expectation that R allele homozygosity at one locus ($[R^{NK}R^{NK} + S^{NKC}X^{NKC}]$, $[S^{NK}X^{NK} + R^{NKC}R^{NKC}]$) will be sufficient to lower blood pressure to levels seen in R allele homozygosity at both loci ($[R^{NK}R^{NK} + R^{NKC}R^{NKC}]$) because R allele homozygosity at any one locus would negate potential synergistic effects on blood pressure irrespective of the other gene's allele status and independent of respective allele specific effects on blood pressure—be it codominant or recessive. Results presented in Table 3 corroborate this notion, showing that rats that are RR homozygous in at least one locus demonstrate blood pressure levels equivalent to subjects that are RR homozygous at both loci. Exclusion of the three groups that are nonhomozygous R allele at either locus, and nonhomozygous S at both loci ($[S^{NK}R^{NK} + S^{NKC}R^{NKC}]$, $[S^{NK}S^{NK} + S^{NKC}R^{NKC}]$, $[S^{NK}R^{NK} + S^{NKC}S^{NKC}]$) was designed as part of the a priori stratification to minimize potentially masking significant gene interaction with "in between" genotypes given that hypertension is polygenic. We note, however, that blood pressure levels of the three groups that are nonhomozygous R allele at either locus are in between and more distant to blood pressure levels of the $[S^{NK}S^{NK} + S^{NKC}S^{NKC}]$ group (Table 3) concordant with the hypothesis of α1NK-NKC loci interaction.

Thus, rat hybrids that are homozygous at one locus of the putative interacting pair in study (α1NK, marked by *D2mgh11,* NKC, marked by *D3mit3*) were contrasted with F2 hybrid rats homozygous for the S allele at both loci. If both loci interactively (epistatically) influence blood pressure, greater statistical association with blood pressure will be detected when analyzed together as interacting genes (digenic analysis, threshold $p < 0.01$) compared with respective gene association with blood pressure when analyzed individually (threshold $p < 0.05$) as reported for diabetes (18). As shown in Table 4, digenic analysis by one-way ANOVA revealed increased susceptibility for SBP ($p = 5.85 \times 10^{-6}$), DBP ($p = 1.98 \times 10^{-5}$), and MAP ($p = 7.73 \times 10^{-6}$). These data suggest that α1NK and NKC loci interactively

Table 1. Correlation of rat chromosome 3 genotype and blood pressure

Locus	Blood Pressure	Genotype				
		RR	RS	SS	ΔBP	*P*
D3rat59	SBP ± SEM	155.8 ± 3.1	157.1 ± 2.3	158.6 ± 2.6	2.8	0.505
	DBP ± SEM	113.3 ± 2.5	112.6 ± 1.9	114.8 ± 2.1	1.5	0.636
	MAP ± SEM	133.5 ± 2.8	133.7 ± 2.1	135.8 ± 2.4	2.3	0.538
	n	23	48	25		
D3rat6	SBP ± SEM	151.8 ± 2.7	159.1 ± 2.4	159.3 ± 2.6	7.5	0.051
	DBP ± SEM	109.8 ± 2.4	114.5 ± 1.9	114.8 ± 2.1	5.0	0.128
	MAP ± SEM	129.5 ± 2.6	135.9 ± 2.1	136.1 ± 2.3	6.6	0.064
	n	26	45	25		
D3rat10	SBP ± SEM	149.8 ± 3.0	159.3 ± 2.3	159.3 ± 2.4	9.5	0.015
	DBP ± SEM	109.0 ± 2.8	114.7 ± 1.8	114.4 ± 2.0	5.4	0.115
	MAP ± SEM	128.0 ± 3.0	136.1 ± 2.1	135.9 ± 2.2	7.9	0.033
	n	22	46	28		
D3mit3	SBP ± SEM	151.1 ± 2.7	158.1 ± 1.9	161.0 ± 3.5	9.9	0.036
	DBP ± SEM	108.4 ± 2.3	113.4 ± 1.6	117.5 ± 2.8	9.1	0.020
	MAP ± SEM	128.5 ± 2.5	134.8 ± 1.8	138.3 ± 3.2	9.8	0.023
	n	22	47	26		
D3rat18	SBP ± SEM	152.4 ± 3.2	158.4 ± 2.2	158.9 ± 2.5	6.5	0.109
	DBP ± SEM	110.1 ± 3.0	114.1 ± 1.8	114.5 ± 2.1	4.4	0.216
	MAP ± SEM	129.8 ± 3.2	135.4 ± 2.0	135.7 ± 2.2	5.9	0.124
	n	22	49	25		
D3rat26	SBP ± SEM	154.5 ± 3.6	159.2 ± 2.3	154.7 ± 1.9	0.2	0.974
	DBP ± SEM	110.4 ± 3.0	115.4 ± 1.8	111.0 ± 1.7	0.6	0.832
	MAP ± SEM	131.0 ± 3.4	136.3 ± 2.1	131.9 ± 1.8	0.9	0.795
	n	17	53	26		
D3rat44	SBP ± SEM	159.2 ± 4.5	157.1 ± 1.9	155.6 ± 2.6	−3.6	0.476
	DBP ± SEM	114.6 ± 3.6	113.8 ± 1.6	111.1 ± 2.1	−3.5	0.395
	MAP ± SEM	135.6 ± 4.1	134.4 ± 1.7	132.4 ± 2.4	−3.2	0.493
	n	19	54	23		
D3rat100	SBP ± SEM	159.2 ± 4.1	156.4 ± 2.0	156.9 ± 2.7	−2.3	0.625
	DBP ± SEM	114.4 ± 3.2	113.3 ± 1.7	112.5 ± 2.2	−1.9	0.614
	MAP ± SEM	135.6 ± 3.7	133.9 ± 1.8	133.6 ± 2.4	−2.0	0.642
	n	21	49	26		

BP, 24-hour average blood pressure in mmHg; SBP, systolic; DBP, diastolic, MAP, mean arterial pressure; SEM, standard error of the mean; RR, homozygous Dahl R alleles; RS, heterozygous; SS, homozygous Dahl S alleles; ΔBP, difference in blood pressure SS-RR; *n,* number of rats in a group; *p, p* value, one-way ANOVA.

influence blood pressure in Dahl salt-sensitive hypertensive rats.

Second, 2 × 2 factorial ANOVA of the entire F2 cohort (no prior stratification) was performed to further validate the putative gene interaction detected by digenic analysis, using blood pressure as the dependent variable. As shown in Table 5, the *p* value for the interaction term is significant supporting interaction between α1NK (first factor) and NKC (second factor) loci in determining the levels of SBP ($p = 0.004$), DBP ($p = 0.008$), and MAP ($p = 0.006$) in the F2 (Dahl S × Dahl R) male cohort.

Molecular Analysis

The molecular dysfunction and mechanistic role of Q276L α1NK variant in the Dahl salt-sensitive

Table 2. Correlation of α1 Na,K-ATPase genotype and blood pressure

Marker		RR	SR	SS	ΔBP	*p*
D2mgh11	SBP ± SEM	151.1 ± 2.2	155.8 ± 2.3	162.5 ± 2.7	11.4	0.006
(α1 Na,K-ATPase)	DBP ± SEM	108.5 ± 1.9	112.9 ± 1.9	116.8 ± 2.3	8.3	0.015
	MAP ± SEM	129.1 ± 2.1	133.2 ± 2.1	138.6 ± 2.5	9.5	0.011
	n	19	45	32		

BP, 24-hour average blood pressure in mmHg; SBP, systolic; DBP, diastolic; MAP, mean arterial pressure; SEM, standard error of the mean; ΔBP, difference in blood pressure SS-RR; *n,* number; *p*, *p* value one-way ANOVA.

Table 3. Blood pressure analysis of α1NK-NKC genotype groups

Genotype Group	*n*	SBP ± SEM	DBP ± SEM	MAP ± SEM
$[R^{NK}R^{NK} + R^{NKC}R^{NKC}]$	6	151.3 ± 6.5	106.8 ± 5.1	128.0 ± 5.9
$[R^{NK}R^{NK} + S^{NKC}R^{NKC}]$	10	151.5 ± 2.0	109.8 ± 2.0	130.0 ± 1.9
$[S^{NK}R^{NK} + R^{NKC}R^{NKC}]$	13	151.5 ± 3.5	109.8 ± 3.2	129.3 ± 3.4
$[S^{NK}R^{NK} + S^{NKC}R^{NKC}]$	20	161.6 ± 3.6	116.0 ± 2.9	137.6 ± 3.3
$[S^{NK}S^{NK} + R^{NKC}R^{NKC}]$	3	149.0 ± 3.5	105.7 ± 0.9	126.0 ± 1.5
$[R^{NK}R^{NK} + S^{NKC}S^{NKC}]$	3	151.7 ± 3.0	107.3 ± 3.8	128.3 ± 3.2
$[S^{NK}S^{NK} + S^{NKC}R^{NKC}]$	17	158.0 ± 2.7	112.6 ± 2.3	134.2 ± 2.6
$[S^{NK}R^{NK} + S^{NKC}S^{NKC}]$	11	151.3 ± 4.6	111.5 ± 3.8	130.5 ± 4.3
$[S^{NK}S^{NK} + S^{NKC}S^{NKC}]$	12	172.2 ± 4.7	125.5 ± 4.0	148.0 ± 4.3

NK: α1Na,K-ATPase; NKC: Na,K,2Cl-cotransporter; SBP, systolic; DBP, diastolic; MAP, mean arterial pressures in mmHg; SEM, standard error of the mean; *n*, number of rats per group.

Table 4. Digenic analysis of α1 Na,K-ATPase and Na,K,2Cl-cotransporter loci

	[R]	[S]	*p* Value
SBP ± SEM	151.3 ± 1.8	172.2 ± 4.7	5.85×10^{-6}
DBP ± SEM	108.7 ± 1.6	125.5 ± 4.0	1.98×10^{-5}
MAP ± SEM	128.9 ± 1.7	148.0 ± 4.3	7.73×10^{-6}
n	35	12	

[R] genotypes: $[RR^{NK} + RR^{NKC}]$; $[RR^{NK} + SR^{NKC}]$; $[RR^{NK} + SS^{NKC}]$; $[SS^{NK} + RR^{NKC}]$; $[SR^{NK} + RR^{NKC}]$; [S] genotype: $[SS^{NK} + SS^{NKC}]$; NK: α1 Na,K-ATPase; NKC: Na,K,2Cl-cotransporter; SBP, systolic; DBP, diastolic; MAP, mean arterial blood pressures in mmHg; SEM, standard error of the mean; *n*, number of rats per group; *p* value, *p* value obtained in one-way ANOVA.

Table 5. 2 × 2 factorial ANOVA determination of α1NK-NKC gene interaction

Dependent Variable	Interaction Term (α1NK × NKC)	
	F	*p* Value
SBP	8.617	0.004
DBP	7.307	0.008
MAP	8.011	0.006

Interaction term *p* value, 2 × 2 factorial ANOVA. The independent variables consisted of indicator variable values (e.g., R allele = 1; S allele = 2) assigned to each rat based on the genotypes for *D2mgh11* (α1NK, factor 1) and for *D3mit3* (NKC, factor 2) markers. 2 × 2 factorial analysis was done independently for SBP, DBP, and MAP.

hypertensive rat model have been elucidated (4,15,23). The genetic evidence presented above strongly suggests the NKC as a putative interacting hypertension susceptibility gene. To support this hypothesis, we investigated potential functionally significant mutation(s) in the Dahl S NKC gene by characterizing both the Dahl S and Dahl R NKC cDNAs completely. Although minor differences were identified between the Dahl S/Dahl R and the reported Sprague-Dawley sequence (14) (Dahl S/Dahl R $\rightarrow$ Sprague Dawley: $T_{160} \rightarrow A_{160}$; $A_{261} \rightarrow R_{261}$; $A_{267} \rightarrow R_{267}$; $A_{333} \rightarrow G_{333}$), no differences were detected within the NKC cDNAs (3.6 kb each), as well as within the alternative spliced exons 4B, 4A, 4F and introns 4 and 5 (1.45 kb each) between the Dahl S and Dahl R rats (10-kb nucleotide sequence; data not shown). This sequence analysis detected 100% amino acid sequence identity between rat and mouse exons 4A and 4F (8) in contrast to previous sequencing data (9).

We then investigated the hypothesis that potential regulatory mutations affecting NKC gene expression levels and/or isoform distribution of the three NKC isoforms could account for the increased Cl^- transport observed in the Dahl S rat. To test this hypothesis, we determined the steady-state levels of the three NKC mRNA isoforms (NKC-B, -A, and -F) in Dahl S and Dahl R rat kidneys at a prehypertensive stage (10 weeks of age; Dahl S, $n = 6$, SBP= 125 $\pm$ 4; DBP = 93.6 $\pm$ 3.5; MAP = 113.2 $\pm$ 0.6 mmHg; Dahl R, $n = 6$, SBP =112 $\pm$ 2; DBP = 92.1 $\pm$ 2.7; MAP = 109.7 $\pm$ 2.9 mmHg). Essentially identical results were obtained when quantitative analysis was done either by RT-PCR at the exponential part of the amplification curve (Fig. 2; see Methods) or classical Northern blot analysis using isoform specific probes (data not shown). As shown in Fig. 2, although steady-state levels of total NKC mRNA are equivalent between Dahl S and Dahl R rat 10-week-old kidneys (Fig. 2a), there is a striking differential pattern of isoform-specific expression levels between Dahl S and Dahl R rat kidneys (Fig. 2b). The Dahl S rat exhibits increased levels of isoform A with a concomitant decrease of isoform F levels when compared to Dahl R (Fig. 2b). Isoforms NKC-B, -A, and -F account for 1.48%, 87.1%, and 11.5%, respectively, of the total NKC mRNA sequences expressed in Dahl S kidney. In contrast, a reversal of isoform trend is detected in Dahl R kidney with isoforms NKC-B, -A, and -F representing 2.44%, 41.2%, and 56.4%, respectively, of total NKC mRNA levels. This finding suggests the existence of structural differences between the Dahl S and Dahl R NKC primary transcripts affecting the alternative splicing pathway and/or precursor mRNA isoform stability resulting in a differential pattern of NKC mRNA isoform expression in Dahl S and Dahl R rats.

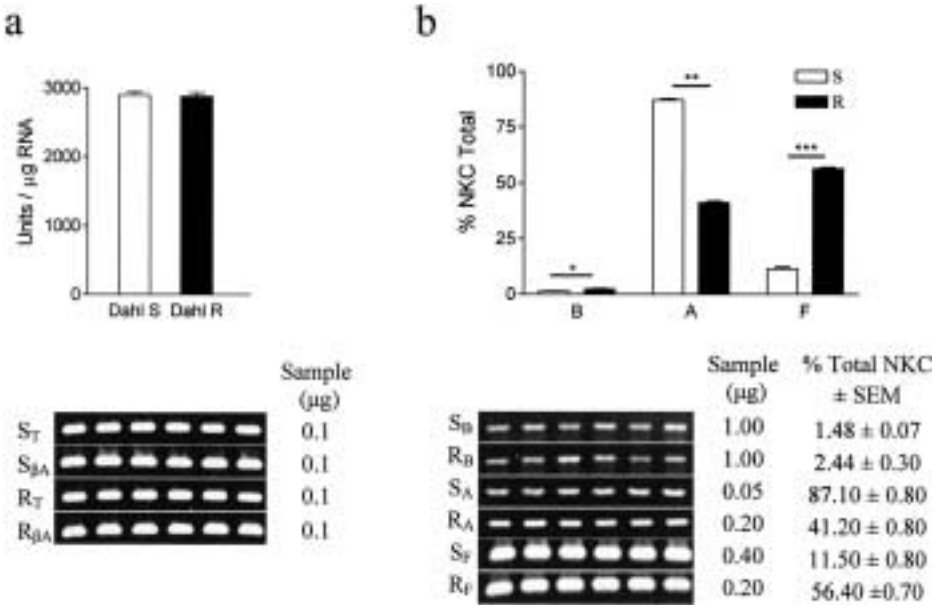

Fig. 2. Differential expression of Na,K,2Cl-cotransporter isoforms, NKC-B, NKC-A, and NKC-F in 10 week-old Dahl S and Dahl R kidneys. (a) Densitometric quantitative analysis of RT- PCR products detect equivalent amounts of total NKC mRNA levels in 10-week-old male Dahl S (S_T) and Dahl R (R_T) kidneys. Data shown are the mean values ($\pm$SEM) of 6 Dahl S and 6 Dahl R kidneys ($p = 0.77$, one-way ANOVA). β-actin RT-PCR products from Dahl S ($S_{\beta A}$) and Dahl R ($R_{\beta A}$) are shown as controls. (b) Densitometric quantitative analysis of RT-PCR products detect different levels of each NKC isoform, NKC-B, NKC-A, and NKC-F, between 10-week-old male Dahl S and Dahl R kidneys resulting in differential isoform ratios. Data shown are the mean values ($\pm$ SEM) of six Dahl S and six Dahl R kidneys. One-way ANOVA statistical analysis $*p = 0.02$; $**p < 0.0001$; $***p < 0.0001$. Different amounts of total kidney RNA samples were used as noted (Sample [μg]) to obtain RT-PCR amplified product at exponential phase (see Materials and Methods for details).

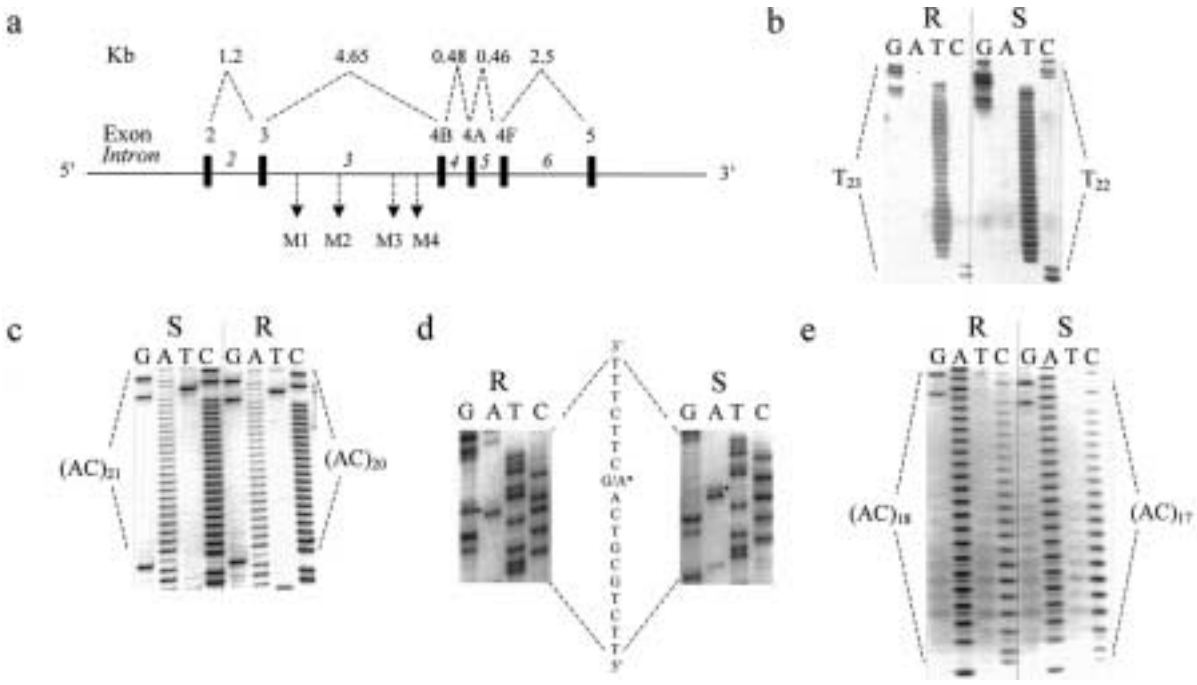

Fig. 3. Nucleotide sequence analysis of NKC gene. (a) Diagram depicting intro/exon organization of the alternative spliced exons (4B, 4A, and 4F) region within the NKC gene. Relative positions of the mutations (M1, M2, M3, and M4) detected in this study are shown within intron 3 (4.65 kb). Nucleotide sequence analysis of intron 3 revealed the following mutations: M1, $(T)_{23}/(T)_{22}$ nucleotide insertion/deletion (Dahl R/Dahl S) at position 1040 in (b); M2, $(AC)_{21}/(AC)_{20}$ dinucleotide insertion/deletion (Dahl S/Dahl R) at position 1987 in (c); M3, G/A nucleotide transition (Dahl R/Dahl S) at position 3285 in (d); and M4, $(AC)_{18}/(AC)_{17}$ dinucleotide insertion/deletion (Dahl R/Dahl S) at position 3852 in (e).

To identify structural differences between Dahl S and Dahl R primary transcripts potentially affecting the alternative splicing pathway we determined the entire nucleotide sequence of introns −2 (1.2 kb), −3 (4.6 kb), −4 (0.45 kb), and −5 (0.47 kb) from Dahl S and Dahl R rat NKC genes (Fig. 3). These introns flank the alternatively spliced exons 4B, 4A, and 4F. Identical nucleotide sequences were observed in introns 2, 4, and 5 between Dahl S and Dahl R rats (data not shown). Four locations showing nucleotide changes were detected in intron 3: 1) a T nucleotide insertion/deletion (Dahl R/Dahl S) at position 1040 3′ to exon 3 (M1; Fig. 3); 2) an AC dinucleotide insertion/deletion (Dahl S/Dahl R) within a small simple repeat at position 1987 3′ to exon 3 (M2; Fig. 3); 3) a G/A nucleotide transition (Dahl R/Dahl S) at position 3285 3′ to exon 3 (M3; Fig. 3); and 4) an AC dinucleotide insertion/deletion (Dahl R/Dahl S) within a small simple repeat at position 3852 3′ to exon 3 (M4; Fig. 3). The immediate 5′-location of intron 3 with respect to exon 4B (the 5′-proximal alternative spliced exon; Fig. 3), makes this region relevant to alternative splicing mechanisms through the alteration of cis-acting regulatory splicing sequences (24) or through induced changes in secondary and/or tertiary structure of the primary transcript which result in altered spliceosome recognition (24).

Discussion

Altogether, our data demonstrate that α1NK and NKC/*D3mit3* loci interactively increase susceptibility to salt-sensitive hypertension in Dahl salt-sensitive hypertensive rats. This interaction accounts for 30.4% of the genetic variance in SBP between Dahl S and Dahl R rats. The identification of the NKC/ *D3mit3* locus as an interacting hypertension susceptibility locus is poignant, given that it would not have been identified by single candidate gene analysis (this study) or by total genome search as previously demonstrated (20, 21). Additionally, the a priori stratification scheme used for digenic analysis is validated, relevant to the analysis of less robust cohorts, such as heterogeneous human populations.

Although the NKC is the closest candidate gene with hypertension pathophysiologic relevance to the *D3mit3* marker, the possibility of another gene locus is not ruled out at the moment. However, the identification of renal NKC molecular variants and altered renal NKC B:A:F isoform ratios in 10-week-old Dahl S and Dahl R rats provide compelling experimental evidence supporting Dahl S renal NKC as the likely candidate gene interacting with Dahl S α1NK to increase susceptibility to salt-sensitive hypertension. This leads us to hypothesize that the inverse renal NKC-isoform ratio in Dahl S rats might

underlie the differential Cl^- transport in the loop of Henle observed previously in Dahl S rats when compared with Dahl R rats (12). This hypothesis is consistent with the views of Plata et al (25) that changes in alternative spliced isoforms provides a mechanism for differential NKC function, and that individual functional integrity as functional K-dependent, bumetanide-sensitive Na,K,2Cl-cotransporters has been demonstrated for all three isoforms using *Xenopus laevis* oocyte expression system (25). We note that although NKC-F isoform was associated with the highest Na^+ and Rb^+ transport activities in *Xenopus* oocytes when expressed singly, resultant Na:Rb(K) ratios were not 1:1; these data could not be confirmed by kinetic studies due to inherent difficulties with the *Xenopus* oocyte system leaving the precise transport kinetic parameters and physiologic roles of NKC-B, -A, and -F isoforms unresolved (25).

Nevertheless, based on the observation that NKC isoforms form multimeric complexes (25), we hypothesize that the physiologic significance of differential NKC isoform ratio observed between Dahl S and R rat kidneys most likely lies in resultant differential oligomerization of NKC cotransporters characterized by increased Cl^- transport (12,13), which contributes to hypertension susceptibility only in the presence of the Q276L Dahl S α1NK variant. This hypothesis remains to be tested in other F2 cohorts, as well as tested for other putative interacting genes such as the CLC-K2 chloride channel also found to exhibit differential expression between the Dahl S and Dahl R rat kidneys (26).

In summary, our data demonstrate the genetic and molecular basis for digenic interaction of α1NK and NKC/*D3mit3* loci to increase susceptibility to salt-sensitive hypertension in the Dahl S rat model. This study validates the gene interaction paradigm for essential hypertension to the level of specific candidate genes beyond hypothetical consideration (27) or putative QTL regions (21). More importantly, this validation provides a framework for investigation of parallel gene interaction mechanisms in human hypertensive patients, as well as in other complex diseases and traits.

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