The Role of Wnk4 in Polygenic Hypertension
A Candidate Gene Analysis on Rat Chromosome 10

Jan Monti, Heike Zimdahl, Herbert Schulz, Ralph Plehm, Detlev Ganten, Norbert Hübner

Abstract—Linkage analyses in experimental crosses of stroke-prone spontaneously hypertensive (SHRSP) and normoten-
sive Wistar-Kyoto (WKY) rats have strongly suggested the presence of quantitative trait loci (QTL) influencing blood
pressure and ACE levels on rat chromosome 10, which have been confirmed in multiple independent studies. Analysis
of the orthologous region on human chromosome 17 also revealed significant linkage to blood pressure in several
populations. Wnk4, a gene previously identified to cause pseudohypoaldosteronism type II, a rare mendelian form of
hypertension, is located on human chromosome 17. The hypothesis has been advanced that molecular variants
of this gene might contribute to common polygenic forms of hypertension, since Wnk4 is located in a region of
conserved synteny that demonstrates an overlap between quantitative trait loci for primary hypertension in humans and
rats. In this report, we describe the confirmation of the blood pressure QTL on rat chromosome 10 by congenic
approaches, spanning the Wnk4 locus. Comparative analysis of the complete coding sequence of Wnk4 in SHRSP and
WKY strains revealed no mutation and demonstrated high conservation between rat and human proteins. Furthermore,
comparison of mRNA levels in the kidney showed no differences between SHRSP and WKY. Additionally, we
excluded a secondary effect of blood pressure on the transcriptional regulation of Wnk4. Our results fail to support a
material contribution of Wnk4 to blood pressure regulation in this model of polygenic hypertension. Thus, Wnk4 is likely
to not represent the underlying disease gene for the QTL captured in chromosome 10 congenic animals. (Hypertension.
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Key Words: blood pressure □ genetics □ hypertension, genetic □ rats, inbred strains □ rats, stroke-prone SHR

Recently, pseudohypoaldosteronism type II, an autosomal
dominant form of hypertension, has been found to be
caused by mutations in the serine/threonine kinase Wnk1 and
Wnk4. A gain-of-function mechanism was postulated for
mutations in both genes either by increased mRNA expres-
sion or premature termination of the protein, respectively. Whereas pseudohypoaldosteronism type II is a rare disorder,
the hypothesis has been advanced that other perhaps more
frequent mutations with less dramatic effects on protein
function may contribute to more common and epidemiolog-
ically more relevant forms of polygenic (essential) hyperten-
sion. This question appeared to be of particular relevance to
hypertension in several substrains of the spontaneously
hypertensive rat (and other hypertensive model strains), in
which previous cosegregation and congenic studies had
demonstrated linkage to the larger “ACE region” (see Rapp
for review),3 which is known to reside in the immediate
vicinity of Wnk4. This chromosomal region shows a highly
conserved synteny between rodents and humans.4 Linkage
data in humans for common familial hypertension has impli-
cated an overlapping region with the rat linkage data at some
distance from the ACE locus—suggesting a potential common molecular cause. To study whether Wnk4 plays a material role in the development of polygenic hypertension, we analyzed chromosome 10 con-
genic rats to confirm the effect of the identified blood
pressure and the presence of quantitative trait loci (QTL)
spanning Wnk4. We have cloned and analyzed the coding
regions in stroke-prone spontaneously hypertensive (SHRSP)
and Wistar-Kyoto (WKY) rats to detect sequence variants
between the two strains. Moreover, we evaluated whether
differential transcriptional regulation of Wnk4 contributes to
the development of elevated blood pressure in this model of
polygenic hypertension and assessed secondary changes in
Wnk4 expression induced by chronic hypertension.

Methods

Animals
All rats were obtained from our colonies at the Max-Delbrück-
Center, Berlin-Buch, Germany. In all groups, we used male rats at 12

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and salt-loaded (BP/SP-1b) blood pressure\textsuperscript{10} and a QTL for two blood pressure QTLs primarily influencing basal (BP/SP-1a) indicated. Subsequently, we have demonstrated that at least interval for initial QTL identification, BP/SP1 (Hilbert, Jacob) is over break point have occurred. To far left, 100:1 odds support bars at both ends indicate chromosome regions where cross-characterization of the congenic segment are indicated. Open some segment of WKY rat being replaced with the homologous markers in centiRad (cR). Solid bar to right represents chromo-

Figure 1. Schematic representation of chromosome 10 congenic W.S10-BP/SP-1b strain. Marker order was derived from radiation hybrid mapping and is in agreement with maps derived from genotyping of chromosome 10 markers in an F\textsubscript{2} WKY×SHRSP\textsuperscript{8–10} Numbers on left indicate distances between markers in centiRad (cR). Solid bar to right represents chromosome segment of WKY rat being replaced with the homologous segment of SHRSP. Polyomorph markers that were used for characterization of the congenic segment are indicated. Open bars at both ends indicate chromosome regions where crossover break point have occurred. To far left, 100:1 odds support interval for initial QTL identification, BP/SP1 (Hilbert, Jacob) is indicated. Subsequently, we have demonstrated that at least two blood pressure QTLs primarily influencing basal (BP/SP-1a) and salt-loaded (BP/SP-1b) blood pressure\textsuperscript{10} and a QTL for determination of ACE plasma levels\textsuperscript{13} are contained within this region of interest. Note that the BP/SP-1b locus captured in the congenic segment does not overlap with the previously described BP/SP-1a congenic locus.\textsuperscript{10}

to 16 weeks of age. Animal experiments were conducted in accordance with institutional guidelines for the care and use of laboratory animals.

Generation of Congenic Rats

We have previously reported on a blood pressure QTL localized on rat chromosome 10.\textsuperscript{9–10} The support interval is given in Figure 1. The SHRSP blood pressure allele between D10 Mgh6 and D10 Mgh4 was transferred onto the WKY background by 10 successive back-crosses starting from F\textsubscript{1} animals. This was accomplished by breeding a male SHRSP with a female WKY. Each subsequent back-cross was performed by mating male rats that have genotypically been confirmed to be heterozygous for the chromosome 10 SHRSP allele with female WKY rats. Breeder animals within each back-cross generation were identified, analyzing multiple SSLP markers within the region of interest, including markers exceeding the 100:1 odds support interval for the localization of the QTL on the telomeric side (see Figure 1). Homozygous W.S10-BP/SP-1b animals were bred according to an analogous breeding scheme described before.\textsuperscript{11} This ensured that on average, >99.8% of the background genome and both sex chromosomes were derived from the WKY recipient. The resulting congenic line was named W.S10-BP/SP1b; W stands for the WKY recipient background, S10 for the SHRSP donor chromosome. BP/SP-1b refers to the precise location of the congenic interval on chromosome 10. The blood pressure QTL BP/SP1 was the first locus identified in SHRSP animals; 1b denotes the telomeric part of this QTL that was used to establish the described congenic line between markers D10 Mgh6 and D10 Mgh4 (Figure 1).

Genotype Determination

DNA was extracted according to standard protocols from tail-tip biopsy specimens. All genetic markers were based on PCR amplification of polymorphic microsatellites as reported previously.\textsuperscript{10,11} Oligonucleotide primer pairs for genetic markers and genotyping protocols were given previously\textsuperscript{10} or can be found at the Rat Genome Database (www.rgd.mcw.edu; accessed March 5, 2003).

Blood Pressure Measurements

Blood pressure was determined by radiotelemetry, as previously described.\textsuperscript{11} Briefly, radiotelemetric pressure transducers were implanted in the abdominal cavity of the rat, with the transducer-connected capillary tubing anchored in the lumen of the abdominal aorta. Animals were allowed to recover for 14 days. Hemodynamic measurements were performed from week 14 to 16 after birth at baseline and during the following 12 days of dietary sodium loading (1% NaCl in drinking water with free access).

Tissue Preparation

All animals were killed by decapitation. Left kidneys were removed, immediately snap-frozen in liquid nitrogen, and stored at \textdegree\textsuperscript{80}C. We extracted total RNA from whole kidneys for further use in quantitative RT-PCR experiments.

Expression Analysis

Quantitative RT-PCR (Taqman) was used to compare mRNA levels of \textit{Wnk4} in kidneys of SHRSP and WKY. DNA-free total RNA (2 \textmu g) was reverse-transcribed with oligo(dT) primers (Gibco-BRL), Superscript II reverse transcriptase (Gibco-BRL), and deoxynucleo-

Cloning and Sequence Analysis

Human \textit{Wnk4} exon-intron structure was determined by using the mRNA-to-genomic alignment program \textit{Spidey} (NCBI, www.ncbi.nlm.nih.gov; human \textit{Wnk4} DNA GenBank accession no.: NM_032387; human BAC clone RP11 to 506G7 gi 21426266 gb AC016889.15). Homologous rat sequences were identified by BLAST search of human \textit{Wnk4}. Primer and probes (\textit{Wnk4} forward: 5[prime]-TCT TCT GAC CCA CGC GTT C-3[prime]; reverse: 5[prime]-TTC TCT TGC CAG CTC CAC A-3[prime]; probe: 5[prime]-6-FAM-TCT GCC GTA CAA AAC AAC ACA GTG TGC-TAMRA-3[prime]) were designed with the use of the program Primer Express 1.0 (PE Applied Biosystems). TaqMan analysis was carried out according to the manufacturer’s instructions, with the use of an Applied Biosys-

Expression levels were calculated relative to WKY animals.

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Phenotype Comparison Between WKY and W.S10-BP/SP-1b Congenic Animals

<table>
<thead>
<tr>
<th>Blood Pressure</th>
<th>WKY (n=12)</th>
<th>W.S10-BP/SP-1b (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure, basal</td>
<td>115.1±2.4</td>
<td>118.0±1.0</td>
</tr>
<tr>
<td>Diastolic blood pressure, basal</td>
<td>80.2±2.4</td>
<td>84.1±0.8</td>
</tr>
<tr>
<td>MAP, basal</td>
<td>95.9±2.4</td>
<td>99.6±0.9</td>
</tr>
<tr>
<td>Systolic blood pressure, NaCl</td>
<td>116.4±2.5</td>
<td>122.1±1.2*</td>
</tr>
<tr>
<td>Diastolic blood pressure, NaCl</td>
<td>79.8±2.6</td>
<td>86.8±1.0*</td>
</tr>
<tr>
<td>MAP, NaCl</td>
<td>96.3±2.6</td>
<td>102.7±1.1*</td>
</tr>
</tbody>
</table>

All blood pressures were obtained at 12 to 16 weeks of age, one day before dietary NaCl loading was initiated (basal), and 12 days after NaCl loading (NaCl), respectively. MAP, mean arterial pressure.

*P<0.05.

Statistical Analysis
All data represented were analyzed with a paired or an unpaired t test. All results are expressed as mean±SEM.

Results

Genetic Characterization of Chromosome 10 Congenic Animals
Transferred SHRSP donor alleles on the recipient WKY background are represented in Figure 1. Genotype analysis with polymorphic microsatellite markers indicated that the W.S10-BP/SP-1b congenic strain encompasses SHRSP alleles between D10 Mgh6 and D10 Mgh4, respectively. The congenic status of the animals was confirmed by genotyping 71 polymorphic microsatellite marker throughout the genome (see supplementary information posted online at http://www.hypertensionahaj.org).

Phenotype Characterization of Congenic Animals
Telemetric blood pressure measurements revealed a significant increase in diastolic and systolic blood pressure after salt loading (Table). The magnitude of this effect was approximately 6 mm Hg. Basal blood pressure values were higher in chromosome 10 congenic animals, but this trend did not reach statistical significance (Table). The strain differences persisted consistently over the whole time period studied and mirrored the observations made at baseline and on day 12 after sodium exposure (data not shown).

Sequence Analysis of Wnk4
Twelve Wnk4 rat exons (exon 1 to 7, 11, 12, 16 to 19) were predicted by BLAST search against the rat trace archive using individual human Wnk4 exons. Corresponding rat trace file sequences were downloaded and aligned to human exons. Conserved splice sites and conserved exon sizes (except for exon 1 and 16) were observed. No orthologous rat trace sequences were identified for human exons 8 to 10 and 13 to 15. Primers were designed from predicted rat Wnk4 sequences, and overlapping PCR products from cDNA in WKY and SHRSP rats were generated for the entire coding region. Sequences were assembled, and 19 exons were identified. The entire open reading frame comprises 3633 nucleotides. Sequence data for the complete coding sequence of Wnk4 have been deposited under Genbank accession no. AY187039. The predicted rat protein shows 93% similarity to the human Wnk4 protein (Figure 2). Comparative sequence analysis between WKY and SHRSP cDNA revealed no differences in the entire coding region.

Kidney mRNA Analysis
Quantitative RT-PCR of kidney Wnk4 mRNA revealed no difference in gene expression between WKY and SHRSP, as shown in Figure 3A. To determine whether Wnk4 transcript levels are influenced by marked chronic blood pressure changes, we measured transcript levels in kidneys from TGR(mRen2)2712 transgenic rats. The markedly elevated chronic arterial blood pressure in these animals had no effect on the transcriptional regulation of Wnk4 compared with WKY normotensive rats. Blood pressure differences and transcript levels are represented in Figure 3B.

Discussion
The mutations initially identified in patients with pseudohypoaldosteronism type II cause either an increase of transcript levels in Wnk1 or a truncated protein in Wnk4, which are believed to result in a gain-of-function in the kidney.1

The colocalization of Wnk4 with blood pressure QTLs for primary hypertension initially identified as the ACE locus on rat chromosome 10 (see Rapp 2000 for review)3,4 and subsequently on human chromosome 17,5,7 along with the speculation that less dramatic mutations in Wnk4 may contribute to polygenic hypertension,1,2 spurred our interest in examining this gene further.

The lack of clear evidence that abnormalities in sodium handling play a prominent pathogenic role in SHRSP hypertension does not detract from the possibility that perhaps more subtle functional mutants of Wnk genes than those operative in pseudohypoaldosteronism type II may contribute to blood pressure regulation, analogous to arguments advanced regarding human essential hypertension.1

The identification of an underlying disease gene(s) in a QTL for blood pressure regulation in rats requires that the biological effect attributed to the identified QTL is confirmed before embarking on laborious efforts to identify the underlying causal genetic variation in the region of interest. We have therefore generated congenic animals encompassing the blood pressure QTL BP/SP-1b on rat chromosome 10. The observed elevated blood pressure in W.S10-BP/SP-1b congenic animals confirms that genetic variation within this region on chromosome 10 contributes to the phenotypic variation attributed to this QTL in SHRSP rats.

With this in mind, we set out to evaluate whether genetic variation at the Wnk4 locus may contribute to the blood pressure increase observed within this QTL. Since most genetic variation will be expressed either by variants affecting the protein sequence or expression levels of a given gene, we have investigated differences in the coding sequence of Wnk4 between SHRSP and WKY and its transcriptional regulation. As indicated above, both mechanisms have been shown to be operative in the Wnk gene family leading to pseudohypoaldosteronism type II.1

Our findings, however, supported by complete cDNA sequence analysis of Wnk4 in WKY and SHRSP, exclude the
presence of any mutation in the coding sequence of this gene. In addition, in the current investigation, no differences in Wnk4 gene expression in the kidney—Wnk4 localizes to the distal nephron segment—between the parental strains were found. The phenotypic analysis of the W.S10-BP/SP-1b congenic animals mirrors our previous observations that the blood pressure QTL identified in this region of chromosome 10 is mainly one that is dependent on salt loading, as shown in F2 cosegregation analysis.8–10 We have therefore evaluated Wnk4 transcriptional regulation also under salt loading to determine whether differences in gene expression may only be visible under this stimulus. To exclude the remote possibility that increased blood pressure values in SHRSP— independent from the investigated QTL—may counterregulate a potential SHRSP allele-specific increase in Wnk4 expression, we investigated the effect of markedly elevated arterial hypertension on Wnk4 gene expression. To this end, we used TGR(mRen2)27 transgenic animals, which have blood pressure values that are comparable to those in SHRSP.12 Again, no differences in transcriptional regulation of Wnk4 could be detected between transgenic and normotensive WKY animals.

**Perspectives**

The speculation is intriguing that genes to which rare single-gene syndromes have been mapped might represent, on the basis of mutations with less severe functional consequences, candidate genes that contribute to common, polygenic manifestations of the same morbid phenotype. Despite the increased odds for such a possibility in the WKY×SHRSP intercross because of prior linkage and confirmation of this QTL in a congenic strain, our data fail to support a role relevant to blood pressure for Wnk4 in this model of polygenic hypertension. The described congenic region contains the ACE locus. We previously demonstrated that ACE plasma levels are genetically determined but are dissociated from
blood pressure regulation in an WKY × SHRSP intercross, making a contribution of ACE plasma levels to blood pressure regulation unlikely. Additional experiments with the established congenic animals will further characterize this QTL and may lead to the identification of the molecular cause underlying blood pressure and ACE plasma level variation.

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References


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