Genetic basis of the impaired renal myogenic response in FHH rats

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The Fawn-Hooded Hypertensive (FHH) rat is a genetic model of hypertension (33) that develops proteinuria, glomerulosclerosis, chronic renal failure, and renal hemodynamics. We previously reported that substitution of a 99.4-Mbp region of Brown Norway (BN) rat chromosome 1 (RNO1) between 258.8 and 261.2 Mbp onto the genetic background of fawn-hooded hypertensive (FHH) rats on autoregulation of renal blood flow (RBF), myogenic response of renal arterioles (AF-art), K+ channel activity in renal vascular smooth muscle cells (VSMCs), and development of proteinuria and renal injury. FHH rats exhibited poor autoregulation of RBF, while FHH.1BN congenic strains with the 2.4-Mbp BN region exhibited nearly perfect autoregulation of RBF. The diameter of AF-art from FHH rats increased in response to pressure but decreased in congenic strains containing the 2.4-Mbp BN region. Protein excretion and glomerular and interstitial damage were significantly higher in FHH than in congenic strains containing the 2.4-Mbp BN region. K+ channel current was fivefold greater in VSMCs from renal arterioles of FHH rats than cells obtained from congenic strains containing the 2.4-Mbp region. Sequence analysis of the known and predicted genes in the 2.4-Mbp region of FHH rats revealed amino acid-altering variants in the exons of three genes: Add3, Rbm20, and Soc-2. Quantitative PCR studies indicated that Mxi1 and Rbm20 were differentially expressed in the renal vasculature of FHH and FHH.1BN congenic strain F. These data indicate that transfer of this 2.4-Mbp region from BN to FHH rats restores the myogenic response of AF-art and autoregulation of RBF, decreases K+ current, and slows the progression of proteinuria and renal injury.

METHODS

General

Experiments were performed in ~200 male FHH and FHH.1BN congenic rats (9–21 wk old) bred and maintained at the University of Mississippi Medical Center, which is fully accredited by the...
American Association for Accreditation of Laboratory Animal Care (AAALAC). All protocols were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center. The rats had free access to food and water. After weaning, the rats were maintained on purified AIN-76 rodent diet containing 0.4% NaCl (Dyets, Bethlehem, PA).

**Protocol 1: Generation of Subcongenic Strains from FHH.1BN Congenic Strain C**

In previous studies, we found that autoregulation of RBF is impaired in FHH rats and transfer of a 4.7-Mb region of RNO1 from BN rats in a FHH.1BN double congenic, presented here as strain C in Fig. 1, restored autoregulation (48). The goal of the present study was to create additional double subcongenic strains to narrow the region of interest further so that positional candidate genes could be prioritized by sequencing and expression studies.

Three male FHH.1BN congenic strain C rats were backcrossed with six female rats from the FHH.1BN congenic strain A to generate an F1 generation, heterozygous for the region of interest and homozygous for BN alleles across the remainder of the genome. From this F1 colony, 18 breeding pairs were set up to generate F2 rats. Each of the F2 animals was genotyped by PCR with a set of 30 polymorphic microsatellite markers equally spaced across the 4.7-Mb region of interest. The genetic distance across the original region was 4.7 Mb, which corresponds to a 5% recombination frequency, and ~700 F2 rats were genotyped to identify the founders for the two new subcongenic lines. The heterozygous founders were backcrossed to FHH.1BN control congenic line A to retain the 99.4-Mbp BN region on RNO1, and the heterozygous progeny were intercrossed to obtain homozygous animals for the two new subcongenic strains D and E.

After narrowing the region of interest by phenotyping strains D and E for autoregulation of RBF relative to the original double congenic strain C and a control FHH.1BN congenic strain A that retains the common 99.4-Mbp region (131.1–230.5 Mbp) of BN RNO1 encompassing the RF-2 region including the Rab38 gene known to prevent bleeding (8) and restore proximal tubular reabsorption of protein (32), we generated a minimal congenic strain to eliminate the possibility that gene-gene interactions are responsible for the restoration of the RBF autoregulation phenotype. This was accomplished by crossing FHH.1BN congenic strain E with FHH rats. The F1 heterozygous rats were intercrossed, and the F2 population was genotyped to find pairs of animals that reverted to the FHH genotype in the 99.4-Mbp RF-2 region but remained heterozygous for the BN genotype in the 2.4-Mbp region of interest from 258.8 to 261.2 Mb of RNO1. Pairs of these animals were intercrossed to generate the FHH.1BN strain F.

**Genotyping.** Genomic DNA was isolated from a piece of tail or the ear using a Direct PCR lysis reagent (Viagen 102-T) and then subjected to PCR amplification using 5'-fluorescent-labeled primers. The PCR reactions were performed in a 10-μl volume and contained 1μl 10× buffer, 100 nM forward and reverse primers, 1.5 mM MgCl2, 250 μM dNTPs, 25 nM Taq polymerase, and 20 ng of genomic DNA. The reactions were denatured at 94°C for 3 min and cycled 25 times at 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. The products were mixed with loading dye (1:1), denatured, loaded on sequencing gels, and read using an ABI 3700 sequencer.
Protocol 2: Autoregulation of RBF in FHH and FHH.1BN Congenic Strains

These experiments were performed in 12-wk-old FHH rats and rats from FHH.1BN congenic strains A, C, D, E, and F (n = 45). The rats were anesthetized with ketamine (30 mg/kg; Phoenix Pharmaceutical, St. Joseph, MO) and Inactin (50 mg/kg; Sigma, St. Louis, MO), and a PE-240 cannula was placed in the trachea to facilitate breathing. The femoral artery and vein were cannulated for intravenous (iv) infusions and measurement of arterial pressure, and a clamp was placed on the aorta above the left renal artery for control of renal perfusion pressure (RPP). A 2-mm ultrasonic Doppler flow probe (Transonic Systems, Ithaca, NY) was placed on the left renal artery to measure RBF. The rats received an iv infusion of 0.9% NaCl solution containing 2% BSA at a rate of 100 μl/min to replace surgical fluid losses. After surgery and a 30-min equilibration period, the rats were acutely volume expanded with a solution of 6% BSA in 0.9% NaCl at a dose of 1 ml/100 g to inhibit tubuloglomerular feedback responsiveness. The celiac and mesenteric arteries were then tied off to raise mean arterial pressure (MAP) to ~150 mmHg, and RBF was measured as RPP was lowered from 140 to 60 mmHg in 10-mmHg increments by adjusting the clamp on the aorta.

Protocol 3: Time Course of Development of Hypertension, Proteinuria, and Glomerular Injury in FHH and FHH.1BN Congenic Strains

These experiments were performed in 9- to 21-wk-old FHH, FHH.1BN control congenic strain A that did not autoregulate RBF and FHH.1BN congenic strains C, E, and F that did autoregulate RBF. Proteinuria was measured at 9, 12, 15, 18, and 21 wk using the Bradford method and BSA as the standard (Bio-Rad Laboratories, Hercules, CA). At each time point, the rats were placed in metabolic cages overnight for determination of protein excretion. At 11 wk of age, some of the rats in each group were anesthetized with isoflurane and telemetry catheters (model TA11PAC40, Data Sciences International, St. Paul, MN) were surgically implanted in the femoral artery with transmitters placed under the skin. MAP was recorded between 9 and 12 AM when the rats were 12, 15, 18, and 21 wk of age. At 21 wk, the rats were anesthetized and the kidneys were flushed with 10 ml of 0.9% NaCl via a cannula placed in the aorta. The kidneys were then collected, weighed, and fixed in a 10% buffered formalin solution. Arterial sections were cut (3 μm) and stained with Masson’s trichrome to determine the degree of glomerular injury and renal interstitial fibrosis. Images were captured using a Nikon Eclipse 55i microscope (Eclipse Ti; Nikon, Melville, NY), and cannulated with glass pipettes. Afferent arterioles were perfused with MEM from the proximal end of the nephron to inhibit tubuloglomerular feedback responsiveness. The afferent arterioles were then tied off to raise mean arterial pressure (MAP) to ~150 mmHg, and RBF was measured as RPP was lowered from 140 to 60 mmHg in 10-mmHg increments by adjusting the clamp on the aorta.

Protocol 4: Myogenic Response in Isolated Afferent Arterioles

The myogenic response was measured in afferent arterioles microdissected from the kidneys of 9- to 10-wk-old FHH rats and FHH.1BN congenic strains E and F. The rats were anesthetized with isoflurane, and the kidneys were removed and placed in ice-cold MEM (GIBCO, Grand Island, NY) containing 5% BSA. Superficial afferent arterioles with an attached glomerulus were microdissected, transferred to a temperature-regulated chamber (37°C) mounted on an inverted microscope (Eclipse Ti; Nikon, Melville, NY), and cannulated with glass pipettes. Afferent arterioles were perfused with MEM from the proximal end. The vessels were imaged using a digital CCD camera (CoolSnap Photometrics, Tucson, AZ), and the images were digitally acquired and vessel diameters were determined using NIS-Elements imaging software (Nikon). Perfusion pressure was set to 60 mmHg, and after a 30-min equilibration period the baseline diameter of the vessel was determined. Perfusion pressure was then increased to 120 mmHg, and after 5 min the diameter of the vessel was redetermined. Additional experiments were performed in afferent arterioles isolated from FHH rats and FHH.1BN congenic strain F before and after removal of Ca2+ from the bath to determine the degree of myogenic tone. Experiments were also performed in vessels isolated from FHH rats and FHH.1BN congenic strains E and F before and after treatment with ibertoxicin (100 nM; Anaspec, Fremont, CA) to determine the influence of the large-conductance K+ channel on the myogenic response.

Protocol 5: Patch-Clamp Experiments

These experiments were performed in VSMCs freshly isolated from renal interlobular arteries microdissected from the kidneys of FHH rats and FHH.1BN congenic strains E and F. These vessels were used as is difficult to microdissect a sufficient number of afferent arterioles in a limited period of time to isolate VSMCs, and we have previously reported that the myogenic response is impaired in isolated interlobular arteries of FHH rats (43). The myogenic response was measured in afferent arterioles isolated from FHH rats and FHH.1BN congenic strains C, E, and F that did not autoregulate RBF. The rats were anesthetized using isoflurane. The kidneys were removed, placed in ice-cold PBS, and renal interlobular arterioles were microdissected. After dissection, the arterioles were digested in a dissociation solution containing 145 mM NaCl, 4 mM KCl, 1 mM MgCl2, 10 mM HEPES, 0.05 mM CaCl2, and 10 mM glucose for 10 min at room temperature. The undigested vessels were pelleted at 1,000 rpm for 5 min, and the supernatant was removed. The VSMCs were incubated in fresh dissociation solution also containing papain (22.5 U/ml, Sigma) and dithiothreitol, 1 mg/ml, for 12 min at 37°C and centrifuged at 1,000 RPM for 5 min. The supernatant was removed, and then the vessels were incubated in fresh dissociation solution containing collagenase (250 U/ml; Sigma), trypsin inhibitor (10,000 U/ml), and elastase (2.4 U/ml) and incubated for 12 min at 37°C. Single cells were released by gentle pipetting of the digested tissue. The supernatant containing VSMCs was collected, and the VSMCs were pelleted by centrifugation. They were then resuspended in fresh dissociation solution and held at 4°C until use in a patch-clamp experiment that was performed within 2–4 h after cell isolation.

Whole cell patch-clamp experiments. K+ currents were recorded from VSMCs using a whole cell patch-clamp mode at room temperature. The bath solution contained (in mM) 130 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, and 10 glucose (pH 7.4), and the pipettes were filled with a solution containing (in mM) 130 K gluconate, 30 KCl, 10 NaCl, 1.8 CaCl2, 1 MgCl2, and 10 HEPES (pH 7.4). The concentrations of EGTA and Ca2+ in the pipette solution were varied to obtain cytosolic free Ca2+ concentrations of 0.1 or 1 μM. The pipette clamp pipettes were constructed from 1.5-mm borosilicate glass capillaries using a two-stage micropipette puller (model PC-87; Sutter Instruments, San Rafael, CA) and heat-poled using a microforge. The pipettes had tip resistances of 2–8 MΩ. The tip of a pipette was positioned on a cell, a 5- to 20-GΩ seal was formed, and the membrane was ruptured by repeated gentle succion with a glass syringe. An Axopatch 200B amplifier (Axon Instruments, Foster City, CA) was used to clamp pipette potential and record whole cell currents. The amplifier output signals were filtered at 2 kHz using an eight-pole Bessel filter (Frequency Devices, Haverhill, MA). The currents were digitized at a rate of 10 kHz and stored on the hard disk of a computer for off-line analysis. Data acquisition and analysis were performed using Clampfit software (version 10.0, Axon Instruments). Outward currents were elicited by 20-mV voltage steps (300-ms duration, 5-s intervals) from ~60 to +120 mV from a holding potential of ~40 mV. Peak current amplitudes were obtained by averaging 5–10 trials. Membrane capacitance was determined by integrating the average capacitance in response to a 5-mV pulse. Peak currents were expressed as current density (pA/pF) to normalize for differences in the size of the VSMCs. In
some experiments, the pipette tips were loaded with a control pipette solution and then back-filled with solution containing 300 mmol/l tetrodotoxin (Anaspec) so that the BKCa current could be measured before and after blockade of the large-conductance BKCa channel.

Single-channel experiments. Single-channel BKCa currents were recorded from VSMCs using inside-out patch-clamp mode at room temperature. The bath solution contained (in mM) 145 KCl, 0.37 CaCl2, 1.1 MgCl2, and 10 HEPES (pH 7.4). The pipettes were filled with a solution containing (in mM) 145 KCl, 1.8 CaCl2, 1.1 MgCl2, and 5 HEPES (pH 7.4). Free cytosolic Ca2+ concentrations were adjusted to 0.1 or 1 μM. The pipettes had a tip resistance of 8–10 MΩ. After positioning of the pipette top on the surface of a cell, a 5-μl seal (5–20 GΩ) was formed by applying light suction. The inside-out patch configuration was achieved by rupturing the membrane with a sudden upward movement of the pipette. An Axopatch 200B amplifier (Axon Instruments) was used to clamp pipette potential and record single-channel currents. Data acquisition and analysis were performed using pCLAMP software (version 7.02, Axon Instruments). Open-state probability (Popen) for single-channel currents, expressed as a percentage of the total recording time in which a channel was open, was calculated using the formula

\[
P_{\text{open}} = \frac{P_{\text{on}}}{P_{\text{on}} + P_{\text{off}}} = \frac{1}{1 + e^{-\frac{V - V_{1/2}}{k}}}
\]

where \(V_1/2\) is the half-maximal voltage and \(k\) is the slope factor.

NPJ is the total recording time. Single-channel currents at a given conductance level, \(j\), represents the sum of the open time at a given conductance level, \(j\), multiplied by the open probability of a given conductance, and \(T\) is the total recording time. Single-channel current recordings at membrane potentials between −60 and +80 mV and at [Ca2+] of 0.1 or 1 μM were used to calculate NPJ.

Protocol 6: Sequencing and Expression Analysis of Genes in the 2.4-Mb Candidate Region

Sequencing of FHH/EurMcwi genomic DNA was performed using an Illumina HiSeq 2000. Sequence reads were aligned to the BN reference genome and single nucleotide variants (SNVs) were called using the CASAVA v1.8.1 program (Illumina, San Diego, CA). The potential functional consequences of variants identified by 10 or more reads with a variant frequency of >40% were predicted with Ensembl Variant Effect Predictor v2.21. Predicted nonsynonymous variants were further analyzed using the Polyphen program (31) to predict the functional consequence to the protein. To confirm that the predicted sequence variants were also captured in the FHH rats and FHH.IBN congenic strains in our colonies, two of the functionally interesting positional candidate genes in the region (Dusp5 and Add3) were resequenced by Sanger sequencing as described before (35). PCR primers were designed to amplify all the exons and introns across the coding regions of these genes from genomic DNA. The primers included M13 tails that allow for direct sequencing of the products using a big dye terminator cycle sequencing kit and an ABI model 3730 automatic sequencer (Applied Biosystems, Foster City, CA). The sequence files were transferred to a UNIX workstation, and base calling sequence assembly and polymorphism detection were performed using a variety of sequence analysis programs, i.e., PHRED, PHRAP, CONSED, and POLYPHRED, looking for stop codons, splice variants, altered start sites, frame shifts, and/or major amino acid substitutions that could alter the function of these proteins.

Quantitative PCR (Q-PCR) assays were also performed to determine whether any of the genes in this region are differentially expressed in renal microvessels. Renal microvessules were bulk-isolated from the kidneys of 10-wk-old FHH rats and FHH.IBN congenic strain F (6 rats/group) using a previously described sieving method (6, 49). The rats were anesthetized with isoflurane. A midline abdominal incision was made, and the abdominal aorta was cannulated. The kidneys were flushed with 5 ml of an ice-cold physiological salt solution followed by 5 ml of a 1% solution of Evans blue to aid in visualizing vessels. The kidneys were removed, and the renal cortex was isolated and placed through a 150-μm sieve that was autoclaved and treated with RNAlater. Glomeruli and tubules passed through the sieve, leaving intact vascular trees on the screen for collection. We have previously reported that this method for isolation of renal microvessules is very effective at removing glomeruli and tubules, and that the vascular preparation is >95% pure with some contamination by adherent proximal tubules. The resulting microvessel preparation was collected in RNAlater and examined under a stereomicroscope for any remaining tubular tissue, which was removed by microdissection before placing of the vessels in TRIzol to isolate the RNA. We previously reported that the residual contamination of bulk isolated microvessules by tubules after microdissection as assessed by measuring expression of the proximal tubular markers, γ-glutamyl transpeptidase, and alkaline phosphatase is negligible (49).

After isolation, the vessels were homogenized using a ground-glass tissue homogenizer in 250 μl TRIzol (Invitrogen), and RNA was isolated. The RNA concentration of the samples was determined using a Synergy 2 plate reader (Biotek, Winoski, VT), and the concentration was adjusted to 1.000 ng/μl. RNA quality was evaluated using the Experion™ System (Bio-Rad). RNA was converted to cDNA using an iScript cDNA synthesis kit (Bio-Rad) using 1 μg of input RNA in a 20-μl reverse transcription reaction. Q-PCR reactions were performed using a CFX96 Real-Time System C1000 Thermal Cycler (Bio-Rad) and normalized to the expression of β-actin. The primers used to amplify the various genes in the region are presented in Table 1. Q-PCR contained Fast SYBR Green Master mix (Thermo Scientific), 0.25 ng of the forward/reverse

### Table 1. Forward and reverse primers used in quantitative PCR experiments to compare the expression of genes in renal vessels of the 2.4-Mbp region of interest in FHH/EurMcwi2 vs. BN rats

<table>
<thead>
<tr>
<th>Genes in the 2.4-Mbp Region</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>RGD1516133 Similar to 60S ribosomal L8</td>
<td>gttggcactcatgctgagg</td>
<td>tcaattctgcccggccgccgc</td>
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<tr>
<td>Soluble X-prolylaminopeptidase 1</td>
<td>actaccgcgccgatcctgagga</td>
<td>gcgcgtctgtgctgctgctgg</td>
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<tr>
<td>MAX interacting 1</td>
<td>cgtcttgctgctgctgctgctg</td>
<td>ggcctacgtgcacagctgctg</td>
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<tr>
<td>Addacin 3 (gamma)</td>
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<tr>
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<tr>
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<td>Survival motor neuron domain</td>
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<tr>
<td>LOC100360511 Hypothetical protein</td>
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<td>cgagtcagtgctgctgctgctg</td>
</tr>
<tr>
<td>Dual-specificity phosphatase 3</td>
<td>cgagtcagtgctgctgctgctg</td>
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<tr>
<td>LOC100360558 Ribosomal S12-like</td>
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<td>β-Actin</td>
<td>cgagtcagtgctgctgctgctg</td>
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BN, Brown Norway rats.

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primers, and 4 ng of cDNA in a total of 25 µl. The thermal profile consisted of 95°C for 10 min, then 40 cycles of 94°C for 15 s, 58°C for 30 s, 72°C for 25 s, and a final melt curve was generated from 65 to 95°C in 0.5°C increments. Reactions without template cDNA were performed to assess nonspecific signals. Each sample was assayed in triplicate.

Statistical Analysis

Mean values ± SE are presented. The significance of the difference in mean values was determined using SigmaStat software and a paired t-test (2 samples) or a one-way or two-way analysis of variance for repeated measures followed by a Holm-Sidak test for preplanned comparisons. *P < 0.05 was considered to be significant.

RESULTS

Protocol 1: Generation of Subcongenic Strains from FHH.1BN Congenic Strain C

A genetic map comparing the regions of BN RNO1 introgressed into the various FHH.1BN congenic strains is presented in Fig. 1. We previously reported that transfer of a 99.4-Mb region of rat RNO1 from BN rats into FHH rat RNO1 restored autoregulation of RBF and reduced proteinuria in FHH.1BN double congenic strain B (24). Subsequently, we developed an additional double congenic strain C and narrowed the region of interest to a 4.7-Mb region on RNO1 (48). In the present study, we created two additional double congenic strains, D and E, that split the 4.7-Mb introgressed BN region on the q terminus of RNO1 in half. Finally, we took strain E in which autoregulation of RBF was restored and backcrossed them with FHH rats and intercrossed the progeny to create a minimal FHH.1BN congenic strain F in which the 99.4-Mb region of BN RNO1 in the Rf-2 region in congenic strains A through E was reverted back to FHH alleles. Overall, a 2.4-Mb region of the BN rat in RNO1, spanning markers D1Rat09 to D1Rat225, was introgressed in FHH.1BN congenic strain F. According to RGD, there are 15 known and predicted genes in this region listed in Fig. 1. Of these, only three genes, i.e., adducin-γ (Add3), dual-specificity phosphatase 5 (Dusp5), and X-prolyl aminopeptidase 1 (Xpnpep1), have any reported influence on cardiovascular function.

Protocol 2: Autoregulation of RBF in FHH and FHH.1BN Congenic Strains

The results of these experiments are presented in Fig. 2. Basal blood pressures measured under Inactin anesthesia were not significantly different between the strains and averaged ~120 mmHg. Baseline RBF measured at this basal blood pressure was also not significantly different between FHH rats and strains A, C, D, E, and F and averaged 7.8 ± 0.7, 6.5 ± 0.7, 9.9 ± 0.6, and 8.8 ± 0.7 ml·min⁻¹·g⁻¹ kidney wt⁻¹, respectively. Control values of RBF measured at a renal perfusion pressure of 100 mmHg were not significantly different between FHH rats and the FHH.1BN congenic strains. Values are means ± SE. The numbers in parentheses indicate number of animals studied per strain. Control values of RBF measured at a renal perfusion pressure of 100 mmHg were not significantly different between FHH rats and the FHH.1BN congenic strains A, C, D, E, and F and averaged 6.7 ± 0.6, 8.2 ± 1.0, 9.8 ± 0.5, 5.8 ± 1.4, 9.8 ± 0.6 and 8.6 ± 0.6 ml·min⁻¹·g⁻¹ kidney wt⁻¹, respectively. *Significant difference from the control value at 100 mmHg within a strain (P < 0.05). †Significant difference in strains C, E, and F from the corresponding value change in FHH rats (P < 0.05).

Protocol 3: Time Course of Development of Hypertension, Proteinuria, and Glomerular Injury in FHH and FHH.1BN Congenic Strains

A comparison of the time course of changes in blood pressure and proteinuria are shown in Figs. 3 and 4. There was a small (10 mmHg) but significant increase in MAP in FHH rats over the course of the study, but not in congenic strains A, C, E, and F (Fig. 3). Protein excretion, (Fig. 4) rose from 42 ± 5 to 259 ± 28 mg/day in FHH rats as they aged from 9 to 21 wk of age. Protein excretion rose to a lesser extent in FHH.1BN congenic strain A that did not autoregulate RBF but has BN in the 99.4-Mbp region of RNO1 which replaces the defective FHH Rab38 gene in the Rf-2 QTL which has been previously reported to contribute to the development of proteinuria in FHH rats by impairing the reuptake of filtered protein (32). A reduction in proteinuria was also seen in congenic strains C and E that did autoregulate RBF but also share the same BN genotype in the Rf-2 region as strain A. Finally, protein excretion was significantly reduced in the minimal FHH.1BN congenic strain F that autoregulates RBF, but does not share the BN genotype in the Rf-2 region. A comparison of the degree of glomerular injury and renal interstitial fibrosis in FHH and the FHH.1BN congenic strains
FHH.1BN congenic strains E and F decreased significantly when transmural pressure was elevated. Blockade of BKCa channels in FHH.1BN congenic strains E and F is presented in Fig. 7. The myogenic response of the afferent arterioles of FHH rats and FHH.1BN congenic strains. Values are means ± SE. Numbers in parentheses indicate number of animals studied per strain. *Significant difference from the control value at 12 wk of age within a strain (P < 0.05). †Significant difference from the corresponding value in FHH rats (P < 0.05).

are presented in Figs. 5 and 6. Representative images of a glomerulus from FHH rats (Fig. 5A) and FHH.1BN congenic strain F (Fig. 5B) are presented. The FHH rats exhibit severe glomerulosclerosis with expansion of the mesangial matrix and increased collagen deposition. The glomerular injury score averaged nearly 3 in 21-wk-old FHH rats, indicating that 75% of the capillary filtration area was lost from most of the glomeruli in the kidney. The degree of glomerular injury was significantly reduced in congenic strains C, E, and F (Fig. 5C) relative to the levels seen in FHH rats and congenic strain A, which does not autoregulate RBF.

Representative images presenting the degree of renal interstitial fibrosis in FHH rats and the minimal FHH.1BN congenic strain F are presented in Fig. 6, A and B, respectively. FHH rats exhibited severe interstitial fibrosis and tubular necrosis, whereas very little fibrosis was found in the renal cortex of FHH.1BN congenic strain F that autoregulates RBF. A comparison of the degree of renal interstitial fibrosis in all of the congenic strains is presented in Fig. 6C. The degree of renal interstitial fibrosis was significantly reduced in the FHH.1BN congenic strains C, E, and F that autoregulate RBF relative to the levels seen in FHH rats but not in FHH.1BN congenic strain A, which did not autoregulate RBF.

Protocol 4: Myogenic Response in Isolated Afferent Arterioles

A comparison of the myogenic response of isolated perfused afferent arterioles of FHH rats and FHH.1BN congenic strains E and F is presented in Fig. 7. The myogenic response of the afferent arterioles of FHH rats was impaired, and the diameter of these vessels increased significantly when transmural pressure was increased from 60 to 120 mmHg. In contrast, the diameter of afferent arterioles microdissected from the kidneys of FHH.1BN congenic strains E and F decreased significantly when transmural pressure was elevated. Blockade of BKCa channels with iberiotoxin (100 nM) restored the myogenic response of the afferent arterioles isolated from FHH rats, but it had no effect on vessels isolated from congenic strains E and F.

The results of experiments to determine the degree of myogenic tone in FHH rats vs. the FHH.1BN congenic strain F are presented in Fig. 8. Afferent arterioles from FHH.1BN congenic strain F constricted in response to an increase in transmural pressure, but the vessel dilated in response to the same stimulus after removal of calcium from the bath, indicating development of substantial myogenic tone. In contrast, the afferent arterioles of FHH rats failed to constrict in response to an increase in transmural pressure, and removal of calcium from the bath had no effect on the response, indicating these vessels fail to develop any myogenic tone.

Protocol 5: Patch-Clamp Experiments

Whole cell studies. A representative tracing of whole cell K+ channel currents recorded from VSMCs isolated from afferent arterioles of FHH rats and FHH.1BN congenic strain F are presented in Fig. 9, A and B. Membrane K+ currents were elicited by a series of 20-mV depolarizing steps (−60 to +120 mV) from a holding potential of −40 mV (Fig. 9B, inset protocol). Cell capacitance was not significantly different among three groups and averaged 16.7 ± 1.5, 15.3 ± 0.83, and 16.5 ± 1 pF in VSMCs isolated from FHH rats and FHH.1BN congenic strains E and F, respectively. K+ channel current was significantly increased at depolarized potentials in renal VSMCs isolated from FHH rats (+40 mV; 19.4 ± 5 pA/pf) by 2.9- and 3.6-fold relative to the currents recorded from VSMCs isolated from FHH.1BN congenic strains E and F (Fig. 9C), respectively. Administration of iberiotoxin (300 nM), a selective BKCa channel blocker, reduced K+ current to a greater extent in VSMCs isolated from FHH rats than that seen in the congenic strain F (Fig. 9D), indicating that increased activity of BKCa channels is primarily responsible for the increased K+ current in VSMCs isolated from FHH rats (FHH rats: +40 mV; before iberiotoxin 19.2 ± 6.2 pA/pF, after iberiotoxin 4.9 ± 1.9

Fig. 3. Comparison of the time course of changes in mean arterial pressure in FHH rats and FHH.1BN congenic strains. Values are means ± SE. Numbers in parentheses indicate number of animals studied per strain. *Significant difference from the control value at 12 wk of age within a strain (P < 0.05). †Significant difference from the corresponding value in FHH rats (P < 0.05).

Fig. 4. Comparison of the time course of changes in proteinuria in FHH rats and the FHH.1BN congenic strains. Values are means ± SE. Numbers in parentheses indicate number of animals studied per strain. *Significant difference from the control value at 12 wk of age within a strain (P < 0.05). †Significant difference from the corresponding value in FHH rats (P < 0.05).
pA/pF; FHH.1BN strain F; +40 mV; before iberiotoxin 4.5 ± 0.6 pA/pF and after iberiotoxin 4.2 ± 0.1 pA/pF).

Single-channel studies. Figure 10A presents representative BKCa single-channel currents recorded from FHH rats and FHH.1BN strain E rats at +40 mV membrane potential and 1 µM free calcium in the bath solution. Single-channel current amplitude plotted as a function of membrane potential is not altered in either strain at 0.1 and 1 µM Ca²⁺ (data not shown).

Fig. 5. Comparison of glomerular injury in FHH rats and the FHH.1BN congenic strains. A and B: representative examples of the degree of glomerular injury seen in 21-wk-old FHH rats (A) and FHH.1BN congenic strain F (B). C: glomerular injury scores measured in all the strains. Values are means ± SE measured from 150 glomeruli (30 glomeruli/rat from 5 rats/strain). *Significant difference from the corresponding value in FHH rats (P < 0.05).

Fig. 6. Comparison of the degree of renal interstitial fibrosis in FHH rats and the FHH.1BN congenic strains. Shown is representative appearance of the renal cortex of 21-wk-old FHH rats (A) and the FHH.1BN congenic strain F (B). Slides were stained with Masson’s trichrome, and the blue color indicates regions of collagen deposition and fibrotic injury. C: renal interstitial fibrosis scores of all strains. Values are means ± SE. Fifteen regions were scored/kidney, and 5 rats/strain were studied. *Significant difference from the corresponding value in FHH rats (P < 0.05).
The BKCa channels exhibited an enhanced \( Np_o \) in response to membrane depolarization or increasing intracellular \( Ca^{2+} \) levels in both FHH and FHH.1BN strain E (Fig. 10B). The \( Np_o \) of the BKCa channel was markedly elevated at all membrane potentials in FHH rats relative to the congenic strain E when channel activity was recorded at a maximal intracellular \( Ca^{2+} \) concentration of 1,000 nM. This difference was even apparent at physiological membrane potentials of \(-40 \) mV (Fig. 10D). \( Np_o \) was also significantly greater in VSMCs isolated from FHH rats than in FHH.1BN rats as recorded at physiological membrane potentials of \(-40 \) mV in the presence of normal resting intracellular \( Ca^{2+} \) concentrations of 100 nM (Fig. 10C).

\section*{Protocol 6: Sequencing and Expression Analysis of Genes in the 2.4-Mb Candidate Region}

Examination of the Rat Genome Database (39) and other sites indicated that there are currently 15 known and predicted genes that map to the 2.4-Mbp region of interest on rat RNO1 (Fig. 1). The results of our comparative sequence analysis identified 3,469 SNVs in FHH/EurMcwi rats vs. the reference BN sequence with a sequence depth of 10 reads or greater and a frequency of 40\% or greater. Of the 3,469 variants identified, 791 are located in 11 of 15 genes of the 2.4-Mbp region of interest, and 20 of these SNVs were found in coding regions of these genes (Table 2). Four genes had no sequence coverage, RGD1561333, Mxi1, LOC100360467, and LOC100360898. The nonsynonymous variants (nsSNVs) found in the coding regions of the Add3 and Soc-2 homolog (Shoc2) genes are predicted to be possibly damaging by Polyphen, while nsSNVs in Rbm20 are predicted to be benign. Of these, two nsSNVs located in Add3 result in a substitution of threonine to serine in exon 2 and lysine to glutamine in exon 13. More than 700 SNVs were identified in the introns of three genes (Xnpp1, Add3, and Rbm20), suggesting a high degree of genetic diversity between FHH and BN rats in these genes, but the functional significance of these sequence variants in the intronic regions remain to be further investigated.

Because of the large number of sequence variants identified in introns and the noncoding regions of many of the genes in the region, we also compared the expression of all 15 genes in renal microvessels isolated from 10-wk-old FHH rats and FHH.1BN congenic strain F (Fig. 11). Only the “Max interactor 1” (Mxi1) and Rbm20 genes were differentially expressed. However, the expression levels of both of these genes are extremely low, with only 0.27 copies of the Mxi1 gene expressed per 1,000 copies of \( \beta \)-actin in strain “F” vs. 0.39 per 1,000 copies of \( \beta \)-actin in FHH. Similarly, 2.52 copies of the Rbm20 gene were expressed per 1,000 copies of \( \beta \)-actin in strain “F” vs. 1.07 copies per 1,000 copies of \( \beta \)-actin in FHH rats.

\section*{DISCUSSION}

The FHH rat is a genetic model that develops mild hypertension, proteinuria and glomerular disease (21, 30, 37, 47) (22, 25–26, 41–42). We have previously reported that the development of proteinuria and glomerular injury in FHH rats is associated with an impaired autoregulation of RBF, GFR, and Pgc (24, 40, 43, 48). More recently, we reported that transfer of 4.7-Mbp region of RNO1 (48) from BN rats into the FHH genetic background restored autoregulation of RBF and attenuated the development of glomerular disease but the mechanism and genes involved are unknown. The purpose of the present study was to narrow the region of interest for the impaired autoregulation of RBF by creating and phenotyping additional FHH.1BN congenic strains and to determine whether the impaired autoregulation of RBF in FHH rats is due to a lack of a myogenic response in the afferent arterioles secondary to an increase in \( K^+ \) channel activity.

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The results of the present study confirm our previous findings that autoregulation of RBF is impaired in FHH rats and it
is fully restored in a dual congenic strain, C, which contains the 99.4-Mbp region of RNO1 from marker D1rat183 to D1rat76 along with a 4.7-Mbp region between markers D1rat376 and D1rat225 (48). To narrow the region further, two new double congenic strains, D and E, were created that cut the 4.7-Mbp region of interest in half. The phenotyping of these strains indicated that the gene(s) responsible for restoration of autoregulation of RBF in FHH.1BN rats is located in the 2.4-Mbp region closer to the q terminus of RNO1 that is introgressed in strain E. To exclude the possibility that the restoration of RBF autoregulation in strain E requires an interaction of BN genes in the two introgressed regions, we created and phenotyped a minimal congenic strain F in which the 2.4-Mbp region of interest alone was introgressed in the FHH genetic background. The results indicate that autoregulation of RBF is also fully restored in the FHH.1BN congenic strain F. This indicates that the gene of interest lies within the 2.4-Mbp interval that contains the 15 genes presented in Fig. 1.

Autoregulation of RBF is mediated by the myogenic response of the afferent arterioles acting in concert with tubuloglomerular feedback. Previous studies by Verseput et al. (44) suggested that TGF feedback responses are intact in FHH rats, which led to the current hypothesis that the impaired autoregulation of RBF might be due to alterations in the myogenic response of the afferent arterioles. To test this hypothesis, we studied whether the myogenic response of the afferent arterioles is altered in FHH rats and restored in FHH.1BN congenic strains. The results indicate that the myogenic response of the afferent arterioles was absent in FHH rats, and the diameter of the afferent arterioles increased in response to elevations in transmural pressure. In contrast, the myogenic response was restored in congenic strains E and F, and the afferent arterioles of these rats constricted in response to the same stimulus. The lack of a myogenic response of the afferent arterioles in FHH rats was not due to elevated vascular resistance or myogenic tone since removal of calcium from the bath had no effect on the diameter of the afferent arterioles in this strain. Nor was the impaired myogenic response secondary to structural changes, since the passive pressure-diameter relationships of the afferent arterioles were identical in FHH rats and the FHH.1BN congenic strain F in Ca^{2+}-free media.

Previous studies by our laboratory and others indicate that the myogenic response of renal and cerebral arteries is associated with depolarization and inhibition of BKCa channel activity (9, 12). Therefore, patch-clamp studies were performed to further explore the mechanism of the impaired myogenic response in FHH rats. The results of these experiments revealed that whole cell K^{+} channel currents are elevated in VSMCs isolated from FHH rats relative to FHH.1BN congenic strains E and F. Single-channel analysis indicated that the elevated K^{+} channel current in FHH rats is associated with a marked increase in the open probability of the BKCa channels compared with those seen in the FHH.1BN congenic strain E. These strain differences were readily apparent when the cells were studied using high intracellular Ca^{2+} concentrations and maximally depolarized potentials to determine whether there was a difference in the Ca^{2+} or voltage sensitivity of the channels between the strains. The results indicate that the BKCa channels in renal VSMCs isolated from both FHH rats and the congenic strains are activated in response to membrane depolarization and elevations in intracellular Ca^{2+} concentration. However, BKCa channel activity was still significantly higher in VSMCs isolated from FHH rats vs. the congenic strain when studied under more physiological conditions using 100 nM intracellular Ca^{2+} concentration and ~40-mV membrane potential. One could argue that baseline BKCa channel activity is very low under these conditions, so it is unlikely to hyperpolarize membrane potential and reduce opening of L-type Ca^{2+} channels. However, previous studies have indicated that since the BKCa channel has a very high

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conductance and the input resistance of VSMCs is so large (38), opening of a very small number of BKCa channels is sufficient to hyperpolarize the membrane potential. Thus in FHH rats the increase in BKCa channel function following elevations in transmural pressure may prevent the VSMC membrane from becoming sufficiently depolarized to increase calcium influx via voltage-gated calcium channels and thus impair the myogenic response. This mechanism is further supported by our observation that blockade of BKCa channels with iberiotoxin normalized the elevated $K_i^{\text{H11001}}$ currents in renal VSMCs of FHH rats and restored the ability of afferent arterioles of FHH rats to constrict in response to elevations in transmural pressure. It has much less effect on $K_i^{\text{H11001}}$ channel activity, and the myogenic response in the FHH.1BN congenic strains in which BKCa channel activity is not elevated. Overall, the results of the renal hemodynamic and patch-clamp studies

Table 2. Single nucleotide variants (SNVs) of genes in the 2.4-Mbp region of interest in FHH/EurMcwi2 vs. BN rat

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene</th>
<th>No. of SNVs</th>
<th>No. of SNVs in Exons</th>
<th>No. of Changes in Amino Acid</th>
<th>Polyphen Prediction</th>
</tr>
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<td>RGD1561333</td>
<td>RGD1561333 Similar to 60S ribosomal L8</td>
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<td></td>
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<tr>
<td>Xpnpep1</td>
<td>Soluble X-prolylaminopeptidase 1</td>
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<td>Mxi1</td>
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<td></td>
<td></td>
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<tr>
<td>Add3</td>
<td>Adducin 3 (gamma)</td>
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<td>7</td>
<td>2</td>
<td>Possibly damaging</td>
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<td>LOC100360467 Max interactor 1-like</td>
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<tr>
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<tr>
<td>Smndc1</td>
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<tr>
<td>Dusp5</td>
<td>Dual-specificity phosphatase 5</td>
<td>14</td>
<td>2</td>
<td>0</td>
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<tr>
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<td>LOC100360558 Ribosomal protein S12-like</td>
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<tr>
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<td>LOC100360174 Binding domain 3-like</td>
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<tr>
<td>Shoc2</td>
<td>Soc-2 (suppressor of clear) homolog</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td>Possibly damaging</td>
</tr>
</tbody>
</table>

N/A, sequence not available.
indicate that the gene responsible for the impaired autoregulation of RBF in FHH rats acts by inhibiting the myogenic response of the afferent arterioles via a mechanism that enhances the activity of BK$_{Ca}$ channels.

We previously suggested that impaired autoregulation of RBF in FHH may trigger the development of proteinuria and glomerular disease by elevating Pgc (48). Thus in the present study we compared the development of proteinuria and glomerular disease in FHH and the congenic strains in which autoregulation of RBF was restored. The present finding that proteinuria was significantly reduced in the FHH.1BN double congenic strains C and E relative to FHH between 9 and 21 wk of age is entirely consistent with this hypothesis. These double congenic strains also exhibited less glomerular injury and renal fibrosis than FHH rats. We also found that FHH.1BN congenic strain A which did not autoregulate RBF was not protected from the development of glomerulosclerosis or renal fibrosis. However, proteinuria was reduced, suggesting other genes and mechanisms may contribute to the protection from the development of proteinuria, but not glomerular injury in this strain. In this regard, the Rab38 gene is located within the 99.4-Mbp region of BN RNO1 introgressed in congenic strains A, B, C, D, and E. We have previously reported that there is a single base pair mutation of the ATG start site of transcription in FHH rats, and they do not express the Rab38 protein (32). Rab38 is involved in membrane trafficking, and loss of this protein mediates the coat color and bleeding disorder phenotype in FHH rats (8) and is thought to increase protein excretion by impairing reuptake and processing of filtered protein in the proximal tubule. Thus replacement of the defective FHH Rab38 gene with the BN wild-type allele likely explains why protein excretion is reduced in FHH.1BN strain A relative to FHH rats even though they do not autoregulate RBF and are not protected from glomerular injury. Correction of the mutated FHH Rab38 allele may also help explain the difference in protein excretion in FHH.1BN congenic strains C and E compared with strain F. Even though they all autoregulate RBF and are all similarly partially protected from the development of glomerular injury, only strain F has been reverted to the defective FHH Rab38 gene. Overall, the present findings are consistent with the view that the development of proteinuria and renal damage in FHH is a result of at least two interacting genetic defects on RNO1, i.e., the defect in the myogenic response resulting in increased transmittal of pressure to the glomerular capillaries to promote glomerular injury and increased filtration of protein acting in conjunction with knockout of the Rab38 gene in FHH rats, which is thought to decrease reuptake and processing of filtered protein in the proximal tubules.

BN rats were chosen as the donor strain for the creation of the consomic and congenic strains as they are the most genetically divergent strain relative to FHH rats with polymorphisms in ~70% of markers across the genome. This facilitated the fine mapping of the genome in regions of interest in our congenic strains. However, the BN rat may have not been the best choice for the substitution since very little is known about steady-state autoregulation of RBF and or the myogenic behavior of renal arterioles of BN rats. Previous investigators have reported that BN rats exhibit impaired high-frequency dynamic autoregulation of RBF relative to spontaneously hypertensive rats (SHR) (46), suggesting a reduced myogenic responsiveness. BN kidneys are also more susceptible to hypertension-induced renal injury when transplanted into SHR (7). On the other hand, BN rats are resistant to renal disease and have an extended life span (27). Mattson et al. (26) have previously done a strain comparison of BN and FHH renal parameters and reported that BN rats are more resistant to the development of proteinuria and renal injury than FHH rats and that substitution of chromosome 1 from BN to FHH rats ameliorates renal injury in FHH.1BN consomic rats. Similarly, a study comparing LN to 10 other normotensive and hypertensive rat strains found that albumin and protein excretion, blood urea nitrogen (BUN), and plasma creatinine are significantly lower in BN than in FHH and Dahl S rats (23). Moreover, the BN rat was simply used as a donor strain for the 2.4-Mbp region of interest on RNO1. Transfer of this region of BN RNO1 into FHH rats improved the myogenic response and corrected autoregulation of RBF in FHH rats. This simply means that other regions of
the genome must be responsible for the differences in autoregulation of RBF previously reported in BN rats relative to SHR.

The results of the present study have established that the gene responsible for altering the myogenic response lies within the 2.4-Mb region of interest of RNO1 containing the 15 known and predicted genes listed in Fig. 1. Of these, only three genes, i.e., Xpnpep1, Add3, or Dusp5, have been reported to affect cardiovascular function and could be considered as potential candidate genes.

Ultimately, the identification of a causal gene requires evidence of a sequence variant that alters the expression or function of protein. Thus comparative sequence analysis was performed in FHH vs. BN rats to determine which of the positional candidate genes might contribute to the impaired myogenic response in FHH rats. More than 700 SNVs in 11 of the 15 genes in the 2.4-Mbp region were identified in FHH/ EurMcwi rats vs. the BN reference sequence. However, only five of these SNVs were predicted to alter amino acids in the coding regions of three genes, Add3, Rbm20, and Shoc2.

Add3 is a membrane cytoskeletal protein that binds calmodulin (16) and is involved in the spectrin/actin network assembly. It serves as a substrate for PKC and Rho kinase (20). There are three forms of adducin; α, β, and γ, or Add1, 2, and 3, which are encoded on separate genes. Adducin proteins must form heterodimers or tetramers to function (14), and it has been shown that Add3 dimerizes with Add1 in the kidney (11). Rbm20 is reported to regulate splicing of titin, which is a sarcomeric protein that determines structure and biomechanical properties of striated muscle (17). Shoc2 is a RAS- and RAF-interacting scaffold protein that positively regulates signaling to ERK1/2 (15).

No studies have yet examined the direct involvement of Add3, Shoc2, or Rbm20 in renal myogenic response or K⁺ channel activity. Add3 has been reported to play a role in NaCl cotransporter (NCC) activity in luminal cells of the distal convoluted tubule by binding to phosphorylation sites on the NCC channel to stimulate NCC activity (10). Knockout of Add3 in mice (34) had no effect on blood pressure or red blood cell (RBC) and platelet function. Mutations in Add1 and 2 have been reported to cosegregate with hypertension (1–3, 13) and renal disease (28–29, 45) in patient populations. However, all of the work on the function of the Add family of proteins has focused on its role in the modulation of Na⁺K-ATPase activity and Na transport in the kidney and in regulation of the size and shape of blood cells rather than on the control of vascular function.

A comparison of the expression of the 15 genes and predicted genes in the region in the renal vasculature of FHH rats and the FHH/1BN congenic strain F indicated only two genes, Mxi1 and Rbm20, were differentially expressed in FHH compared with FHH/1BN strain F at 10 wk of age, although neither gene has any known action on vascular function. In addition, the expression of both of these genes was very low in the renal vasculature, <2.5 copies/1,000 β-actin copies, making it less likely that either of these genes is responsible for the impaired myogenic response of FHH rats.

**Perspective**

This study indicates that substitution of a 2.4-Mbp region of BN RNO1 restores the myogenic response in FHH/1BN congenic rats and that the mechanism of the impaired vascular reactivity in FHH is associated with an increase in BKCa activity inafferent arterioles. Three of the 15 genes in this region have sequence variants that could potentially alter the function of the proteins (Add3, Shoc2, and Rbm20), and two (Mxi1 and Rbm20) are differentially expressed in renal vessels isolated from FHH vs. that seen in congenic strain F. While mutations in the Add3 gene appear the most interesting at this time, further work is needed to determine which of these genes is responsible for altering the myogenic response in FHH rats and the mechanisms involved.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


