Mutation of SH2B3 (LNK), a Genome-Wide Association Study Candidate for Hypertension, Attenuates Dahl Salt-Sensitive Hypertension via Inflammatory Modulation


Abstract—Human genome-wide association studies have linked SH2B adaptor protein 3 (SH2B3, LNK) to hypertension and renal disease, although little experimental investigation has been performed to verify a role for SH2B3 in these pathologies. SH2B3, a member of the SH2B adaptor protein family, is an intracellular adaptor protein that functions as a negative regulator in many signaling pathways, including inflammatory signaling processes. To explore a mechanistic link between SH2B3 and hypertension, we targeted the SH2B3 gene for mutation on the Dahl salt-sensitive (SS) rat genetic background with zinc-finger nucleases. The resulting mutation was a 6-bp, in-frame deletion within a highly conserved region of the Src homology 2 (SH2) domain of SH2B3. This mutation significantly attenuated Dahl SS hypertension and renal disease. Also, infiltration of leukocytes into the kidneys, a key mediator of Dahl SS pathology, was significantly blunted in the Sh2b3em1Mcwi mutant rats. To determine whether this was because of differences in immune signaling, bone marrow transplant studies were performed in which Dahl SS and Sh2b3em1Mcwi mutants underwent total body irradiation and were then transplanted with Dahl SS or Sh2b3em1Mcwi mutant bone marrow. Rats that received Sh2b3em1Mcwi mutant bone marrow had a significant reduction in mean arterial pressure and kidney injury when placed on a high salt diet (4% NaCl). These data further support a role for the immune system as a modulator of disease severity in the pathogenesis of hypertension and provide insight into inflammatory mechanisms at play in human hypertension and renal disease. (Hypertension. 2015;65:00-00. DOI: 10.1161/HYPERTENSIONAHA.114.04736.) • Online Data Supplement

Key Words: hypertension ■ immune system ■ kidney ■ lymphocytes ■ rats

Cardiovascular disease was the underlying cause of 1 of every 3 deaths in the United States in 2010.1 Cardiovascular disease also causes major morbidity. Hypertension is a major risk factor for cardiovascular disease; it affects roughly 1 of 3 adults in the United States.1 The cause for >90% of cases of hypertension is unknown, contributing to the statistic that only ≈50% of patients with hypertension have their blood pressure controlled through current therapies. This observation highlights the importance of continued research on mechanisms involved in blood pressure regulation and the pathophysiology of hypertension to develop new therapies for the treatment of this common risk factor for cardiovascular disease.

Recent genome-wide association studies (GWASs) in humans have nominated candidate genes for hypertension, a genetically complex disease trait.2 The general functions of such candidate genes are often unknown and not intuitively linked to hypertension. Exploring the mechanistic relationship between the candidate genes and hypertension may lead to the discovery of novel pathways involved in blood pressure regulation, which may identify therapeutic targets for the treatment of hypertension. The gene SH2B3 (LNK) has been linked to human hypertension and renal disease by GWASs.3,4 This gene encodes SH2B adaptor protein 3 (SH2B3, LNK), an intracellular adaptor protein shown to play a major role in hematopoiesis and cytokine signaling.3 From N-terminal to C-terminal, SH2B3 contains a proline-rich protein interaction site, a pleckstrin homology domain, a Src homology (SH2) domain, and many putative tyrosine phosphorylation sites.5 Although most functional testing of SH2B3 has been performed in hematopoietic cells and endothelial cells, it is expressed in many other tissues, such as the kidney, brain, and various muscle types. Because of its potential role in inflammatory pathway regulation, we sought to explore the role of SH2B3 in a well-established model of hypertension, the Dahl salt-sensitive (SS) rat.
Recent experimental and clinical evidence has shown a role for immune-mediated mechanisms in the pathogenesis of hypertension. In the Dahl SS rat, the development of hypertension and renal disease is accompanied by a significant accumulation of T lymphocytes and macrophages in the kidneys. Pharmacological or genetic immunosuppression inhibits the infiltration of immune cells in the kidneys and significantly blunts the hypertension and kidney injury. Because of SH2B3’s expression in immune cells (notably T cells and macrophages) and the importance of inflammatory signaling in the Dahl SS phenotype, we aimed to assess whether the GWAS-nominated gene, SH2B3, may be linked to hypertension via inflammatory mechanisms. Targeting of SH2B3 for mutation on the Dahl SS genetic background allowed us to assess its importance in Dahl SS pathology. Zinc-finger nuclease–mediated genetic mutation led to a 6-bp deletion in a highly conserved region of SH2B3. This deletion is predicted to affect a phosphotyrosine-binding site in the SH2 domain, a region that facilitates signal transduction via interactions with phosphorylated tyrosine residues on other proteins. Because there is significant attenuation of hypertension and kidney injury in the Sh2b3em1Mcwi mutant rats, experiments were performed to confirm that alterations in immune cells directly play a role in the blunted phenotype.

Methods

Animals

All animal breeding and experimental procedures were performed at the Medical College of Wisconsin after protocols approved by the Institutional Animal Care and Use Committee. Methods about the generation of the Sh2b3em1Mcwi mutant rat, phenotyping, and statistical analyses are provided in the online-only Data Supplement.

Results

6-bp Deletion Present in the Mutant SH2B3 mRNA Does Not Affect Gene Expression, But Is Predicted to Alter a Highly Conserved Protein Sequence

To test the contribution of SH2B3 to SS hypertension, zinc-finger nucleases were designed to target a highly conserved sequence within the SH2B3 gene. We validated the mutation in RNA by designing polymerase chain reaction primers to amplify the mutation site of SH2B3 in cDNA. After polymerase chain reaction amplification, the resulting amplicons in cDNA were separated on a polyacrylamide gel. Ethidium bromide detection revealed that the Sh2b3em1Mcwi mutant transcript to be smaller than the Dahl SS transcript, indicating a deletion within the sequence (Figure 1A). The polymerase chain reaction products were then cloned and sequenced, confirming a 6-bp deletion at the mutation site (Figure 1C). This mutation did not alter the mRNA expression of SH2B3 in the kidney or peripheral blood mononuclear cells compared with the Dahl SS (Figure 1B); however, the mutation was predicted to modify protein sequence. As shown in Figure 1D, the mutated amino acids (red) are evolutionarily conserved among all 83 analyzed vertebrate species (bottom) and are located in the phosphopeptide-binding site in the SH2 domain (top, JAK2 shown as a representative phosphotyrosine-binding partner). The native proline–leucine–glutamic acid sequence is replaced with a single glutamine in the Sh2b3em1Mcwi mutant rat, resulting in modification of the phosphotyrosine-peptide binding pocket.

Mutation of SH2B3 Expands Immune Cell Compartments and Increases the Percentage of T Regulatory Cells Within the T Cell Population

Because of SH2B3’s importance in immune cell development, differentiation, and signaling, changes in global immune system characteristics were determined in Sh2b3em1Mcwi mutant rats maintained on 0.4% NaCl or fed 4% NaCl for 3 weeks (n=4–7/group). Compared with the Dahl SS rats, the Sh2b3em1Mcwi mutants had significantly greater spleen weights (2.4±0.2 versus 1.5±0.1 g) and showed a significant increase in the number of total peripheral blood mononuclear cells (PBMCs; 14.7±0.6 versus 5.9±0.9 10⁶/mL), including T cells (7.0±0.4 versus 3.3±0.5 10⁶/mL), and B cells (4.0±0.3 versus 1.0±0.2 10⁶/mL; Figure 2A). High salt diet had no effect on these values. Independent of the increase in the total number of immune cells, mutation of SH2B3 significantly increased the percentage of T regulatory cells (Tregs; CD4+CD25+FOXP3+) within the T cell population in both the circulation (5.2±0.3 versus 3.5±0.1%) and spleen (4.9±0.1 versus 4.0±0.1%; Figure 2C and 2D) compared with the Dahl SS controls. Additionally, high salt diet caused a significant increase in the percentage of Tregs within splenic T cells in the Sh2b3em1Mcwi mutant (5.9±0.2 versus 4.9±0.1%), but not the Dahl SS.

Sh2b3em1Mcwi Mutant Rats Have Attenuated Hypertension and Albuminuria

Perturbations to the immune system have been shown to affect blood pressure and renal function in Dahl SS rats. These characteristics were assessed in the Sh2b3em1Mcwi mutant rat to determine if mutation of SH2B3 affected the progression of Dahl SS pathology. Figure 3 shows the differences in blood pressure and albumin excretion rate between age-matched Dahl SS and Sh2b3em1Mcwi mutants (n=6–8/group) maintained on 0.4% NaCl chow or fed 4.0% NaCl chow for 21 days. There was no difference in mean arterial pressure between Dahl SS and Sh2b3em1Mcwi mutants maintained on 0.4% NaCl chow (127.7±2.0 versus 127.5±1.5 mm Hg); but, the Sh2b3em1Mcwi mutants fed 4.0% NaCl chow for 21 days had significantly lower blood pressure than did Dahl SS rats on the same diet (135.7±1.1 versus 175.5±7.9 mm Hg). The albumin excretion rate was significantly lower in the Sh2b3em1Mcwi mutants compared with the Dahl SS rats at days 0 (3.6±0.3 versus 20.0±2.1 mg/d), 10 (9.4±2.4 versus 101.1±15.4 mg/d), and 21 (30.3±7.0 versus 104.9±8.9 mg/d) of 4.0% NaCl diet. These data indicate that mutation of SH2B3 significantly attenuates Dahl SS hypertension and renal disease.

Sh2b3em1Mcwi Mutant Rats Have Reduced Renal Damage and Blunted Infiltration of Immune Cells in the Kidney

Figure S1 in the online-only Data Supplement illustrates the renal histological changes because of increased salt intake of the Dahl SS and Sh2b3em1Mcwi mutant rats (n=5–6/group). As expected, the Dahl SS rats developed substantial renal injury.
in response to high salt diet, including significant glomerular damage and outer medullary damage (blocked and dilated tubules). These features were not present in the Sh2b3em1Mcwi mutants fed high salt. In confirmation of our previous reports,9,13,14 Dahl SS rats fed 4.0% NaCl chow for 21 days had a significantly greater infiltration of leukocytes (CD45+ cells) into the kidneys (3.92±0.42 versus 2.13±0.36 10⁶/kidney), including T cells (CD3+ cells; 0.42±0.04 versus 0.21±0.03 10⁶/kidney), compared with Dahl SS rats maintained on 0.4% NaCl (Figure 4). In contrast to the observations in the Dahl SS, no differences in immune cell infiltration in the kidney were observed between Sh2b3em1Mcwi mutants fed 0.4% NaCl and those fed 4.0% NaCl chow. Furthermore, Sh2b3em1Mcwi mutant rats on high salt had significantly less renal infiltration of immune cells than Dahl SS rats on high salt (1.84±0.30 versus 3.92±0.42 10⁶/kidney).

**Mutation of SH2B3 Does Not Affect Vascular Reactivity**

As mentioned above, SH2B3 is expressed in endothelial cells. To determine whether preserved vascular function contributes to the attenuation of hypertension in the Sh2b3em1Mcwi mutant rats, we measured vascular reactivity in resistance vessels isolated from rats fed 0.4% NaCl or 4.0% NaCl diet. We postulate that any changes in vascular function because of the mutation of SH2B3 would be ubiquitous. Therefore, we used mesenteric arteries as proxies to resistance vessels throughout the body. Figure S2 illustrates vascular reactivity of small mesenteric arteries as measured by wire myography (n=4–7/group). Regardless of rat genotype (Dahl SS or Sh2b3em1Mcwi) or diet (0.4% NaCl or 4.0% NaCl), there were no significant differences among the groups in vasoconstriction to serotonin (5-HT) or vasodilation to the endothelial-dependent dilator acetylcholine (ACh).

**Mutation of SH2B3 Attenuates Hypertension and Albuminuria via the Hematopoietic Compartment**

Because of the reduced accumulation of immune cells in the kidneys and the blunted pathology in Sh2b3em1Mcwi mutant rats, experiments were performed to determine if discrepancies in immune cell function between Dahl SS and Sh2b3em1Mcwi mutants could account for the attenuated phenotype. The role of immune cells in the disease phenotype could be tested by transferring immune cell compartments between the genotypes via bone marrow transplants. After total body irradiation, Dahl SS and Sh2b3em1Mcwi mutant rats (n=5–6/group)
underwent bone marrow transplantation, receiving either Dahl SS or Sh2b3em1Mcwi mutant bone marrow. Figure 5 shows that regardless of the recipient’s genotype, those rats that received the Sh2b3em1Mcwi mutant bone marrow had a significant attenuation of hypertension during the last week of the high salt treatment compared with the rats that received the Dahl SS bone marrow. Likewise, once placed on high salt chow, significant elevation of mean arterial pressure compared with low

Figure 2. Quantification of peripheral blood mononuclear cells (PBMCs; A) of age-matched Dahl salt-sensitive (SS) and Sh2b3em1Mcwi mutant rats. Representative scatter plots from flow cytometric analysis displaying CD4+CD45+ PBMCs from Dahl SS and Sh2b3em1Mcwi mutant rats (B). Percent expression of circulating (C) and splenic (D) T regulatory cells (CD4+CD25+FOXP3+) within the T cell population of Dahl SS and Sh2b3em1Mcwi mutant rats maintained on 0.4% NaCl (low salt, LS) or 4.0% NaCl (high salt, HS) chow. *P<0.05 vs values obtained on 0.4% NaCl chow. †P<0.05 vs Dahl SS on the same diet.

Figure 3. Mean arterial pressure (A) and albumin excretion rate (B) of age-matched Dahl salt-sensitive (SS) and Sh2b3em1Mcwi mutant rats maintained on 0.4% NaCl chow or fed 4.0% NaCl chow for 21 days. *P<0.05 vs values obtained on 0.4% NaCl chow. †P<0.05 vs Dahl SS on the same diet.

Figure 4. Quantification of total leukocytes (CD45+ cells; A) and T lymphocytes (CD3+ cells; B) in the kidneys of age-matched Dahl salt-sensitive (SS) and Sh2b3em1Mcwi mutant rats maintained on 0.4% NaCl chow or fed 4.0% NaCl chow for 21 days. *P<0.05 vs values obtained on 0.4% NaCl chow. †P<0.05 vs Dahl SS on the same diet.
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Immune-Mediated Hypertension and Kidney Injury

Salt values was delayed in rats with Sh2b3em1Mcwi mutant bone marrow than in rats with Dahl SS bone marrow. Rats with Sh2b3em1Mcwi mutant bone marrow also had attenuated albuminuria compared with the Dahl SS counterparts on days 14 and 21 of high salt chow. Collectively, these data indicate that the Sh2b3em1Mcwi mutant bone marrow cells are contributing to the attenuation of hypertension and renal damage.

Majority of PBMCs in the Transplanted Rats Are of Donor Genotype

After 21 days of high salt treatment, the bone marrow transplant groups were euthanized for tissue analysis. Having measured significant differences in the phenotypes among the bone marrow transplant groups, experiments were performed to determine the effectiveness of the bone marrow transplants in each group. To do so, RNA was extracted from PBMCs and converted to cDNA. The cDNA samples were analyzed via real-time polymerase chain reaction with genotype-specific primers. The primers were designed to determine the presence of Dahl SS and Sh2b3em1Mcwi mutant genotypes in the circulating immune compartment, as shown in Figure S3. The 3’ end of the Dahl SS forward primer contains the 6 bases that are not present in Sh2b3em1Mcwi mutant transcript. Likewise, the Sh2b3em1Mcwi mutant forward primer excludes the 6 deleted bases in its sequence. The genotype-specific forward primers share a reverse primer. By normalizing the genotype-specific Ct values to Ct values obtained from an SH2B3 primer set flanking the mutation (amplifies both forms of SH2B3 mRNA), the percentage composition of Dahl SS PBMCs and Sh2b3em1Mcwi mutant PBMCs could be determined in the transplanted rats (n=4–6; Figure S3, bottom). The slight detection of the Sh2b3em1Mcwi mutant transcript in the Dahl SS control is because of nonspecific amplification at high Ct values and has little effect on the overall trend of chimeric bone marrow reconstitution.

Discussion

SH2B3 was shown to be associated with hypertension and renal disease in human GWASs via a single nucleotide polymorphism in the coding region of the pleckstrin homology domain.14 The aim of our study was not to recapitulate the GWAS single nucleotide polymorphism, but to explore the role of SH2B3 in hypertension and renal disease. Therefore, the coding region of the SH2 domain was ultimately targeted for mutation on the Dahl SS genetic background because (1) this region was determined to be the optimal site for successful mutation using zinc-finger nuclease technology and (2) the SH2 domain is well characterized for binding partners and function, information that could guide future molecular studies in this model. The 6-bp deletion in the Sh2b3em1Mcwi mutant rat resulted in a significant attenuation of Dahl SS pathology, showing a direct role for SH2B3 in hypertension and kidney injury. Although protein modeling predicts altered functionality of the SH2 domain in the mutant rats, the exact molecular changes to protein function (loss of function, gain of function, etc) are unclear.

Testing of vascular reactivity revealed no differences in dilatory or contractile ability between the 2 strains, suggesting that alterations in intrinsic vascular function do not contribute to the attenuated hypertension in the Sh2b3em1Mcwi mutant rats.
though release of local vasoactive agents (possibly by immune cells) during the progression of disease could potentiate hypertension. Furthermore, Dahl SS or Sh2b3\textsuperscript{em1Mcwi} mutants that underwent total body irradiation and were repopulated with Sh2b3\textsuperscript{em1Mcwi} mutant bone marrow had significantly lower blood pressure and albuminuria than the rats repopulated with Dahl SS bone marrow. These studies indicate an attenuation of the Dahl SS pathology via alterations to the immune cell compartment, although the exact mechanism(s) remain elusive.

SH2B3 is a negative regulator of lymphopoiesis, as indicated by the enhanced production of lymphocytes in Lnk\textsuperscript{−/−} mice\textsuperscript{15} and reduced lymphopoiesis in mice overexpressing SH2B3 (LNK).\textsuperscript{16} We show that mutation of SH2B3 augments lymphocyte development, yet attenuates hypertension. This may seem counterintuitive when considering the abundance of data that indicate a pathogenic role for the immune system in hypertension. We have shown, through numerous experimental approaches, that immunosuppression attenuates Dahl SS hypertension.\textsuperscript{9–11} Many other laboratories have reported the same phenomenon in their respective models of hypertension as well.\textsuperscript{8} Therefore, one might expect that expansion of the immune system would exacerbate hypertension. Interestingly, the kidney seems to be the main site of deleterious inflammation during the pathogenesis of Dahl SS hypertension, incurring significant infiltration of macrophages and T lymphocytes in the renal interstitium.\textsuperscript{9,14} The Sh2b3\textsuperscript{em1Mcwi} mutant rats on a high salt diet have blunted renal infiltration of immune cells and attenuated renal disease and hypertension. The trafficking of immune cells from the bloodstream into tissue involves cell–cell interactions with the endothelium.\textsuperscript{17} SH2B3 has been shown to modulate hematopoietic cell adhesion to vascular adhesion molecule 1.\textsuperscript{18} Mutation of SH2B3 may inhibit pathways that regulate the trafficking of immune cells to target organs, such as the kidney, thus protecting them from deleterious inflammation.

Another plausible mechanism of blunted pathology in the Sh2b3\textsuperscript{em1Mcwi} mutant rats involves Tregs. Tregs are a subtype of CD4+ T cells that suppress innate and adaptive immune signaling.\textsuperscript{19} Currently, the most discriminatory marker for Tregs is the transcription factor forkhead box P3 (FOXP3), an essential component of Treg differentiation in the thymus.\textsuperscript{20} Another characteristic often associated with Tregs is the constitutive expression of interleukin 2 receptor α-subunit (CD25) on the cell surface, although it is postulated that mature CD4+FOXP3+CD25− cells are dormant Tregs that upregulate CD25 expression on homeostatic expansion and activation.\textsuperscript{21} We detect both CD25+ and CD25− Tregs in the Dahl SS and Sh2b3\textsuperscript{em1Mcwi} mutant rats. The balance between Tregs and proinflammatory T cell subsets is important in maintaining immune homeostasis and a deficiency of Tregs leads to autoimmune disease.\textsuperscript{22}

Limited experimental studies suggest a therapeutic role for Tregs in hypertension. Increasing the global expression of Tregs among immune cells via adoptive transfer of exogenous Tregs blunted vascular inflammation and attenuated angiotensin II–induced hypertension and vascular disease in mice.\textsuperscript{23} Apart from the increase in total T cells, mutation of SH2B3 selectively increases the percentage of Tregs within the splenic and circulating T cell populations. Moreover, high salt diet further increases this selective expression of splenic Tregs in the Sh2b3\textsuperscript{em1Mcwi} mutant rats. Considering immune homeostasis as a balance between pro and anti-inflammatory signaling, the increased ratio of Tregs to proinflammatory T cells may provide additional protection from deleterious chronic inflammation. The ratio of Tregs/proinflammatory T cells in the circulation has been shown to decrease in patients with rheumatoid arthritis,\textsuperscript{24} a condition known to be mediated by the immune system. Furthermore, this ratio has been shown to decrease in diseases of supposed inflammatory mediation, such as minimal change nephrotic syndrome\textsuperscript{25} and acute coronary syndrome.\textsuperscript{26} In the case of hypertension, increased Treg/proinflammatory T cell ratios, as seen in the Sh2b3\textsuperscript{em1Mcwi} mutant rats, may suppress tissue inflammation and attenuate disease.

Though T cells play a major role in the development of Dahl SS hypertension, the exact mechanisms responsible for hypertension and organ damage are unknown. By no means should the influence of other inflammatory cell types be disregarded when considering the development of hypertensive pathology. It is known that there is much crosstalk among cells capable of inflammatory signaling. SH2B3 functions in many signaling pathways that are responsible for a multitude of inflammatory activity.\textsuperscript{1} Although the mutation in SH2B3 resulted in a reduction of Dahl SS pathology via immune cells, the mechanism(s) responsible remain elusive. Furthermore, the expression pattern and function of SH2B3 in many tissues and cell types remain unclear. Although we detected SH2B3 mRNA in renal cortical and outer medullary tissue, the effects of its mutation on renal physiology are unknown, much less the innate role of SH2B3 in kidney function. It is possible that mutation of SH2B3 alters blood pressure regulation and renal function via nonimmune mechanisms in the mutant rat. Further studies will need to determine the signaling pathways affected by the mutated SH2 domain of SH2B3 to elucidate downstream targets that result in the attenuation of hypertension and kidney injury. Because of the strong association between SH2B3 and human hypertension and renal disease, the Sh2b3\textsuperscript{em1Mcwi} mutant rat will allow for further dissection of inflammatory mechanisms involved in these pathologies.

**Perspectives**

These studies provide experimental validation of the association between SH2B3 and human hypertension and renal disease described in GWASs. A role for inflammation in the pathogenesis of hypertension is becoming widely accepted, as much data from a variety of experimental models support this hypothesis.\textsuperscript{6–8} These studies indicate that inflammatory pathways could become therapeutic targets for the treatment of human hypertension. Elucidating molecular mechanisms by which SH2B3 modulates Dahl SS hypertension will give insight into possible mechanisms at play in the human condition.

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Disclosures

None.

References


Novelty and Significance

What Is New?

Mutation of SH2B adapter protein 3 (SH2B3) significantly attenuated Dahl SS hypertension via immune cell function, which provides experimental validation of the association between SH2B3 and hypertension proposed in human genome-wide association studies.

What Is Relevant?

Treatment of hypertension is often not optimal. The studies in this article, along with many others, highlight the immune system as a possible therapeutic target, although much work still remains to elucidate inflammatory pathways that mediate hypertension.

Summary

SH2B3, a genome-wide association study candidate for hypertension and renal disease, encodes an intracellular adaptor protein that functions in many signaling cascades. With no understanding of its association to hypertension, we sought to explore its role in the Dahl SS pathology. Mutation of the highly conserved SH2 domain in the SH2B3 protein resulted in a significant attenuation of hypertension and renal disease. Further experimentation indicated a direct role for immune cells in the altered pathology, although the mechanisms remain unclear.
Mutation of \textit{SH2B3 (LNK)}, a Genome-Wide Association Study Candidate for Hypertension, Attenuates Dahl Salt-Sensitive Hypertension via Inflammatory Modulation

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ONLINE SUPPLEMENTAL MATERIAL

Mutation of SH2B3 (LNK), a GWAS candidate for hypertension, attenuates Dahl SS hypertension via inflammatory modulation

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DETAILED METHODS

Animals. The Sh2b3\textsuperscript{em1Mowi} mutant rats were created using ZFN technology as previously described.\textsuperscript{1-3} ZFNs were designed to target exon 2 of the rat \textit{SH2B3} gene, where each member of a heterodimeric ZFN pair binds to the underlined sequence on opposite strands (CTCCCCCATCCACT\underline{tg}aatGTGGAGCAGCCTGTG). Among several mutant founders, one founder animal harboring a 6 base-pair deletion mutation of 5'-\textit{CACTTG}-3' in exon 2 was used for subsequent phenotyping. Experiments were performed using age-matched Dahl salt-sensitive (SS/JrHsdMowi) rats and \textit{SH2B3} mutant (SS-Sh2b3\textsuperscript{em1Mowi}) rats. Rat breeders and weanlings were fed purified AIN-76A rodent diet (Dyets Inc.) containing 0.4% NaCl.

Protein modeling. The modeled JAK2 interaction with \textit{SH2B3} was created by replacing SH2-B of the Protein Databank file 2hdx with modeled \textit{SH2B3} in YASARA\textsuperscript{4}, followed by energy minimization with the AMBER03\textsuperscript{5} force field in an aqueous environment (water and NaCl). The location of the rat mutation was visualized and imaged with YASARA.\textsuperscript{6} Sequences for \textit{SH2B3} were obtained from NCBI and aligned with ClustalW.\textsuperscript{7}

Confirmation of \textit{SH2B3} mutation. RNA was isolated using RNaseasy Plus kits (Qiagen). cDNA was synthesized using 1 \(\mu\)g RNA with cDNA synthesis kits (BioRad). \textit{SH2B3} specific primers were designed to amplify either on the mutation site or around the mutation site. The primer sequences used are as follows:
- Dahl SS-specific \textit{SH2B3} forward – AGCGCTCCCCCATCCACTTG
- Dahl SS-specific \textit{SH2B3} reverse - GTGAGGAGGATCCATTTCATAG
- Sh2b3\textsuperscript{em1Mowi} mutant-specific \textit{SH2B3} forward – TCCAGCGCTCCCCCATCCATGG
- Sh2b3\textsuperscript{em1Mowi} mutant-specific \textit{SH2B3} reverse - GTGAGGAGGATCCATTTCATAG
- All \textit{SH2B3} forward – CAGTGAGGAAGGGAAAAGGG
- All \textit{SH2B3} reverse – GATATGCTCCGCCACTTCC
- \(\beta\)-actin forward – GCCCTAGACTTCGAGC
- \(\beta\)-actin reverse – CTTTACGGATGTCAACGT

RNA was isolated from Dahl SS or Sh2b3\textsuperscript{em1Mowi} mutants, reverse transcribed, and amplified using Platinum Taq polymerase (Life Technologies) for 30 cycles using the “All \textit{SH2B3}” primers listed previously. The PCR products were loaded onto a gradient 4-12% polyacrylamide gel (Bio-Rad) and visualized using ethidium bromide staining. The amplicons were also cloned into the pCR2.1-TOPO vector (Life Technologies) and individual clones were prepared and sequenced using M13 forward and reverse primers as described previously.\textsuperscript{1} Real-time PCR (qPCR) was performed on an ABI 7900HT sequence detection system using SYBR Green detection reagent.

Surgical preparation. At the age indicated, rats were deeply anesthetized with a mixture of ketamine (75 mg/kg ip), xylazine (10 mg/kg ip), and acepromazine (2.5 mg/kg ip), with supplemental anesthesia administered as needed. RenaPulse catheters (Brantree Scientific) were placed in the femoral artery and tunneled subcutaneously to the back of the neck. Alternatively, telemetry transmitters (Data Sciences International) were placed subcutaneously with the catheter inserted in the carotid artery. Both
methods result in the direct measurement of arterial pressure in awake, freely-moving rats. All rats were kept on heating pads throughout the procedure and analgesics and antibiotics were administered to prevent pain and infection.

**Blood pressure measurements and urine collections.** The effects of high NaCl intake on blood pressure were assessed in one of two ways:

1) At 9 weeks of age, untreated Dahl SS and Sh2b3^em1Mcwi mutant rats were switched from 0.4% NaCl chow to 4% NaCl chow (both chows were purified AIN-76A rodent diet from Dyets, Inc.). At 10.5 weeks of age, the rats underwent surgery for catheter implantation in the femoral artery. After 5 days of recovery, blood pressure was measured via a pressure transducer connected to the femoral catheter. Systolic and diastolic pressures were recorded once a min for 3 hrs every day for 7 days. The average measurements from those recordings were used to calculate the daily mean arterial pressure (MAP). Overnight urine collections were performed while the rats were maintained on 0.4% NaCl chow and on days 10 and 21 of 4% NaCl chow.

2) Dahl SS and Sh2b3^em1Mcwi mutant rats that underwent bone marrow transplantation (BMT) were instrumented with telemetry transmitters with the catheter placed in the carotid artery two weeks after BMT. Blood pressure was continuously monitored for 5 days while the rats were fed 0.4% NaCl chow and for the following 21 days while the rats were fed 4.0% NaCl chow. Overnight urine collections were performed while the rats were maintained on 0.4% NaCl chow and on days 7, 14, and 21 of 4% NaCl chow.

**Peripheral blood mononuclear cell isolation.** Heparinized arterial blood (2 ml) was mixed 1:1 with Dulbecco’s phosphate buffered saline (DPBS, 2.7 mM KCl/1.5 mM KH2PO4/137.9 mM NaCl/8.1 mM Na2HPO4-7H2O). The mixture was layered over 3 ml Histopaque-1083 in a 15 ml conical tube. After centrifugation (400g x 30 min at RT), the mononuclear cell layer was extracted and washed in DPBS. Cells were resuspended in DPBS containing 2% FBS and 2mM EDTA, and the cell concentration was determined by counting on a hemocytometer.

**Kidney mononuclear cell isolation.** Mononuclear cell fractions were isolated from kidneys with an approach based on a previously-described protocol. Rats were anesthetized with pentobarbital sodium (50 mg/kg ip) and the kidneys were flushed with heparinized (5%) saline. After removing the renal capsule, the left kidney was minced and teased apart with the plunger of a 1 ml syringe in RPMI 1640 containing 2 mM L-glutamine, 20 mM HEPES, 10% FBS, and collagenase type IV (0.1%). The solution was incubated for 30 min at 37°C with periodic shaking. The kidney homogenate was then filtered through a 70µm cell strainer and washed with DPBS/2% FBS/2 mM EDTA at 4°C at 250g x 5 min. The pellet was resuspended in 10 ml washing buffer and passed through a 40µm cell strainer. After centrifugation (250g x 5 min) the pellet was resuspended in 3 ml FBS (10 mM EDTA), layered over Histopaque-1083, and centrifuged at 19°C at 400g x 30 min. The mononuclear cell layer resting above the Histopaque was removed, counted on a hemocytometer, and washed for flow cytometric analysis.

**Histological analysis.** Kidneys were collected for histological analysis as previously described. Tissue was fixed with 10% paraformaldehyde, paraffin embedded, cut
in 4 µm sections, mounted, and stained with Masson’s trichrome. Images of slides were taken at different magnifications using a Nikon E400 microscope fitted with a Spot Insight camera. The outer medullary cast percentage was determined with color inclusion via MetaMorph software. Individual glomeruli (roughly 40 per rat) were evaluated using the semi-quantitative index method of Raij et al. Glomeruli were scored from 0 (best) to 4 (worst) based on glomerulosclerosis and mesangial expansion as previously described. The histological scoring was performed by an observer blinded to the treatment (low or high salt) and strain (Dahl SS or Sh2b3em1Mowil mutant).

**Flow cytometric analysis.** Mononuclear cells from blood and kidneys (10^6) were resuspended in 100µl buffer (DPBS/0.5% BSA/2mM EDTA) with appropriate amounts of antibody. If intracellular staining was necessary, cells were incubated with Fixable Viability Dye eFluor 450 (Ebioscience) for 30 minutes in DPBS. After washing, cells were stained with the Foxp3/Transcription Factor Staining Buffer Set (Ebioscience) according to the manufacturer’s protocol. The extracellular antibodies APC-anti-CD3, FITC-anti-CD4, PE-anti-CD45R, PE-anti-CD8, and FITC-anti-CD11b, were all purchased from Becton Dickinson. PE-Cy7-anti-CD8, PerCP-eFluor 710-anti-CD3, and APC-anti-FOXP3 were purchased from Ebioscience. PE-Cy7-anti-CD45, FITC-anti-CD8, and APC-Cy7-anti-CD4 were purchased from Biolegend. Cell viability was assessed using DAPI (Sigma). Cells were initially analyzed by flow cytometry (LSRII; Becton Dickinson) using FACSDiva software (Becton Dickinson). Further analysis was performed using FlowJo software. To determine total leukocyte (CD45+) number in the kidney, a “cell gate” was placed on the FSC vs SSC scatter plot of each kidney sample to exclude debris. After dead cell exclusion via DAPI, the percent of CD45+ cells within the live “cell gate” was applied to the total cell count obtained on the hemocytometer for each kidney, resulting in total leukocyte counts per kidney. From these values, subsequent amounts of CD45+ cell types (e.g. CD3+. CD8+, etc) in each kidney could be determined.

**Bone marrow transplantation.** At 7 weeks of age, Dahl SS or Sh2b3em1Mowil mutant rats underwent total body irradiation (TBI) at a dose of 11 Gy at a rate of 1.83 Gy/min. Within 2 hours of TBI, rats received ~0.4 ml DPBS containing either Dahl SS or Sh2b3em1Mowil bone marrow cells via tail vein injection. The bone marrow cells from each genotype were collected from four femora of untreated rats and diluted in 6 ml of DPBS. Two weeks after bone marrow transplantation (BMT), rats were instrumented with radiotelemeters to record blood pressure. Three weeks after BMT, rats were switched to high salt (4.0% NaCl) diet for 21 days. Urine collections were performed at days -1, 7, 14, and 21 of high salt treatment. Following the protocol, rats were euthanized and tissues were collected for further analysis.

**Vascular reactivity testing.** Third-order resistance arteries were identified and isolated from the mesenteric bed, cleaned of connective tissue, and hung on a wire myograph (Danish Myo Technology, Aarhus, Denmark) as previously described. Arteries were bathed in warmed physiological salt solution (119.0 mM NaCl, 4.7 mM KCl, 1.6 mM CaCl2, 1.18 mM NaH2PO4, 1.17 mM MgSO4, 24.0 mM NaHCO3, 5.5 mM dextrose and 0.03 mM EDTA), pH 7.4, and aerated with a 95% O2/5% CO2 mixture. A passive tension of 2 mN was applied, and vessels equilibrated for a period of 1 hr. Following
stabilization, phenylephrine (PE, 10 µM) was added to the bath and when contraction was maximal and stable, acetylcholine (ACh, 10 µM) was added to assess vessel quality. Vessels were included only if contraction reached at least 7 mN and endothelium-dependent dilation exceeded 50%. For vasodilation studies, vessels were preconstricted with 10 µM PE and dilation-response curves to ACh (1nM to 10 µM) commenced once a stable maximal contraction had been achieved. Dilation values are demonstrated as a percent of maximal contraction to PE ± SEM. Additionally, contractile dose-response curves were performed for serotonin (5-HT, 1 nM to 10 µM). Following each contractile or dilatory treatment, vessels were washed 3 or more times in fresh PSS, until baseline had been reestablished. Statistical analyses, including calculation of logEC50 values, were performed using Prism software (GraphPad Software, Inc., La Jolla, CA).

**Statistics.** Data are expressed as the mean ± one standard error. Data were assessed for significance using a t-test, a one-way repeated measures analysis of variance (ANOVA) with a Holm-Sidak post-hoc test, or a two-way repeated measures ANOVA with a Holm-Sidak post-hoc test, as appropriate. A value of p<0.05 was considered significant.
SUPPLEMENTAL REFERENCE LIST


**Figure S1:** Light microscopy of trichrome stained sections of renal outer medulla (A, magnification 100x) and renal cortex (B, magnification, 400x) of Dahl SS and Sh2b3<sup>−/−</sup> mutant rats maintained on 0.4% NaCl chow or fed 4.0% NaCl chow for 21 days. Quantified percentage of renal outer medulla consisting of protein casts (C) and glomerular injury score (D). * indicates p<0.05 vs. values obtained on 0.4% NaCl chow, † indicates p<0.05 vs. Dahl SS on the same diet.
FIGURE S2: Vascular dilation in response to acetylcholine (A) and vascular contraction in response to serotonin (B) of mesenteric resistance arteries from age-matched Dahl SS and Sh2b3<sup>em1Mcwi</sup> mutant rats maintained on 0.4% NaCl chow or fed 4.0% NaCl chow for 21 days. * indicates p<0.05 vs. values obtained on 0.4% NaCl chow, † indicates p<0.05 vs. Dahl SS on the same diet.
Figure S3: Real-time PCR forward primers were designed to distinguish between expression of wild-type (Dahl SS) SH2B3 and mutant SH2B3 (top). The genotype-specific primers shared a reverse primer. Real-time PCR of PBMC cDNA shows recapitulation of Dahl SS and Sh2b3\textsuperscript{em1Mcwi} mutant bone marrow in the BMT studies (bottom).