PAPPA2 IS LINKED TO SALT-SENSITIVE HYPERTENSION IN DAHL S RATS

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Running title: Pappa2 is a candidate gene in Dahl S rats

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Abstract
A 1.37 Mbp region of chromosome 13 was previously identified by exclusion mapping which was consistently associated with a reduction of salt-induced hypertension in the Dahl salt-sensitive (SS) rat. This region contained five genes which were introgressed from the salt-insensitive Brown Norway (BN) rat. The goal of the present study was to further narrow that region to identify the gene(s) most likely to protect from salt-induced hypertension. The studies yielded a subcongenic SS rat strain containing a 0.71 Mbp insert from BN (26-P strain) in which salt-induced hypertension was reduced by 24 mmHg. The region contained two protein-coding genes (Astin1 and Pappa2) and a microRNA (miR-488). Pappa2 mRNA in the renal cortex of the protected 26-P was 6-10 fold greater than in SS fed a 0.4% NaCl diet but was reduced to levels observed in SS when fed 8.0% NaCl diet for 7 days. Compared to brain nuclei (NTS, RVLM, CVLM) and the adrenal gland, Pappa2 in the renal cortex was the only gene found to be differentially expressed between SS and 26-P and that responded to changes of salt diet. Immunohistochemistry studies found Pappa2 localized in the cytosol of the epithelial cells of the cortical thick ascending limbs. In more distal segments of the renal tubules, it was observed within tubular lumens and most notably bound to the apical membranes of the intercalated cells of collecting ducts. We conclude that we have identified a variant form of Pappa2 that can protect against salt-induced hypertension in the Dahl S rat.

Key words: Dahl S rats, genetics, congenic, kidney, Pappa2, Astin1, miR-488, renal collecting ducts, intercalated cells, thick ascending limbs, Na excretion, ENaC
Introduction

The world-wide prevalence of essential hypertension, reported as 26.4% of the adult population, represented nearly 1 billion people in 2000 and is predicted to rise to 1.56 billion by 2025\(^8,\)\(^{20}\). Essential hypertension is a multifactorial polygenic disease determined by an interplay of genes and environment\(^7,\)\(^{18},\)\(^{24},\)\(^{28}\). Genome-wide association studies (GWAS) have found that individual genes can account for only a small percent of the blood pressure (BP) phenotype\(^{11,\)\(^{24},\)\(^{28}\). Despite the polygenic nature of the disease, the vast heterogeneity of genetic backgrounds, and more than 29 GWAS associated polymorphisms, BP in large portions of the population is affected by common stimuli of dietary salt. Enhanced sensitivity of BP to salt intake is present in nearly half of Americans who are afflicted with hypertension and nearly 75% of African American hypertensive patients\(^1,\)\(^{36,\)\(^{40}\). Salt intake is directly correlated with the risk of developing hypertension in both experimental animal models and in human populations\(^23\). There is strong evidence that sensitivity to dietary salt is a highly heritable trait\(^{15,\)\(^{32}\) although the genetic and physiological mechanisms underlying BP salt-sensitivity in essential and most forms of experimental hypertension remain elusive.

The inbred Dahl salt-sensitive (SS) rat has been among the most widely studied heritable models to explore the mechanisms underlying BP salt-sensitivity\(^7,\)\(^{32}\). SS rats recapitulate, in an accelerated way, the progression of hypertension, as exemplified in the African American population and this model has provided key insights into the pathophysiological mechanisms and the genetic complexity underlying salt-sensitivity\(^7,\)\(^{23}\). Congenic mapping studies in which genomic elements of the salt-resistant Brown Norway (BN) rat were introgressed into the isogenic background of the
SS rat identified regions within eight to ten different chromosomes that contribute to BP salt-sensitivity in the SS rat\textsuperscript{26,27}. Within chromosome 13 alone, four non-overlapping genomic regions were identified that significantly influenced BP salt-sensitivity in the SS rat\textsuperscript{27}. One of these regions contained a 13.11 Mbp segment of the BN salt-resistant strain genome consistently associated with attenuation of salt-induced hypertension in male rats\textsuperscript{6}. To further narrow this region, 13 overlapping subcongenic strains were developed by multiple backcrossing, which by exclusion mapping identified a 1.37 Mbp region containing five genes that were consistently associated with a reduction of salt-induced hypertension\textsuperscript{6}. The goal of the present study was to experimentally confirm this region and identify the gene(s) most likely to protect from salt-induced hypertension. An inbred subcongenic strain containing a 0.71 Mbp insert from the BN rat (26-P interval) was developed which contained two protein-coding genes (\textit{Astn1} and \textit{Pappa2}) and a microRNA (\textit{miR-488}). This 0.71 Mbp insert reduced salt-sensitivity by 24 mmHg mean arterial pressure in SS rats. \textit{Pappa2} was uniquely differentially expressed in the renal cortex comparing SS and 26-P rats, and the only gene affected by salt diet. The data indicate that \textit{Pappa2} is a novel candidate gene in the development of salt-sensitive hypertension.

\textbf{Materials and Methods}

\textbf{Experimental animals and strain nomenclature} All rats were bred and housed in an American Association for Accreditation of Laboratory Animal Care accredited animal care facility at the Medical College of Wisconsin with free access to water and to a custom AIN-76A purified rodent chow (0.4\% NaCl; Purified AIN-76; Dyets, Inc.,
Bethlehem, PA). This same chow formulation, but with 8% NaCl, was used in studies where the diet was changed for 7 or 14 days. All experimental procedures were approved by the Institutional Care and Use Committee of the Medical College of Wisconsin.

Previous exclusion mapping identified a 1.37 Mbp region containing five genes (Fam5b, Astn1, Pappa2, Rfwd2, Tnr) that influenced the mean arterial pressure (MAP) by 42 mmHg in SS rats fed a high salt diet\(^6\). For the present study, the protected congenic strain 26-M spanning the 80.99 to 84.61 Mbp interval was back-crossed to SS and the F1 generation rats were genotyped with 59 microsatellite markers covering the interval with markers deposited into the Rat Genome Database (http://rgd.mcw.edu/). Recombinant rats were selected as breeders for the establishment of the six overlapping subcongenic strains as shown in Figure 1 (flanking markers for each congenic strain displayed). The corresponding strain names are as follows: 26-N: SS.BN-(D13Hmgc 1048–D13Hmgc 664)/Mcwi; 26-O: SS.BN-(D13Hmgc 1048–D13Hmgc1045)/Mcwi; 26-P: SS.BN-(D13Hmgc1048-D13Hmgc1050)/Mcwi; 26-Q: SS.BN-(D13Hmgc1041-D13Hmgc664)/Mcwi; 26-R: SS.BN-(D13Hmgc885–D13Hmgc664)/Mcwi; 26-S: SS.BN-(D13Hmgc1041- D13Hmgc885)/Mcwi.

**Phenotyping** 5-6 week old male rats of each strain (SS, 26-N, 26-O, 26-P, 26-Q, 26-R, 26-S) were surgically prepared for the measurement of mean arterial pressure (MAP) and heart rate (HR) using radiotelemetry techniques, as we have described\(^9,14\). Rats were anesthetized with 2.5% isoflurane (remainder oxygen) and a gel-filled catheter implanted in the right carotid artery and the attached transmitter (model TA11PA-C40, Data Systems International, Minneapolis, MN) secured subcutaneously between the
scapulae. Post-operative administration of buprenorphine provided analgesia and the
rat was returned to the home cage for a 5-7 day recovery period. Following recovery,
rats were placed in metabolic cages without restricting food or water for an overnight
urine collection to measure baseline albuminuria and then returned to the home cage
for measurement of blood pressure. Blood pressure was measured daily from 9:00 a.m.
to 12 p.m. during a period when access to the recording room was restricted. It was this
blood pressure phenotype that was used for narrowing of the region in the development
of these subcongenic strains. After measurements were made for three consecutive
control days, the dietary intake of NaCl was increased from 0.4% to 8% with blood
pressure measurements made for 14 days of high salt with a second overnight
collection of urine following the measurements on the last day of 8.0% NaCl diet. From
urine collected, albumin was quantified using an Albumin Blue 580 (Molecular Probes)
fluorescence assay.

**RNA expression** In separate groups of rats of all strains, kidney cortex and outer
medulla tissue, and adrenal gland as well as left ventricle, thoracic aorta, abdominal
muscle and liver were collected from 7-8 weeks old rats on 0.4% salt diet or on 8% salt
diet for seven days, snap frozen in liquid nitrogen and stored at -80°C. At the same
time, whole brain tissue was collected and embedded in OCT through cold 2-
methybutane and stored at -80°C. Thick slices (~1mm) of brain tissue were made using
an Alto adult rat brain matrix form caudal to rostral beginning with the cervical spinal
cord through the pontomedullary junction. Circular tissue punches (blunted 18.5 gauge
needle) were then taken from the ventral midline from the first four slices to sample
three nuclei of the brainstem known to be involved in the autonomic control
cardiovascular and pulmonary functions. The locations of these regions were
determined using the rat brain stereotaxic map as we have recently described\textsuperscript{31}.
Specifically, these regions were the nucleus tractus solitarius (NTS), rostral ventrolateral
medulla (RVLM) and the caudal ventrolateral medulla (CVLM).

Tissues were collected after only seven days of high salt to minimize the effects
of the hypertension itself upon gene expression. RNA from collected tissue was
extracted using Trizol reagent (Life Technologies), quantified by a Nanodrop
spectrophotometer, and the quality of each sample assessed with Agilent 2100
BioAnalyzer to ensure an RNA Integrity Number > 8\textsuperscript{25}. A two-step qRT-PCR approach
was carried out for the mRNA quantification of expression and 18s used for internal
normalization. For cDNA synthesis, a RevertAid First Strand cDNA Synthesis Kit
(ThermoSci.) was used. The two step qRT-PCR analysis for microRNA was carried out
using the modified real time PCR method described previously\textsuperscript{38}. RUN6B was used for
the experiments in Figure 3 for internal normalization but later experiments used 5s
rRNA.

For amplification, rat \textit{Pappa2} primers were purchased from Qiagen (Cat. No.
QT00457387). The amplified PCR product from the kidney cortex had the following
sequence:
\begin{verbatim}
GTATCTGTGAACTTCTATGTGGATGCACACCACCATGGGCAAGCCACTGGAAAA
AGG AGGCTCTCGGAGAACCTGGTAACTCACAGGTCTGCATCCA.
\end{verbatim}
The primer sequences for \textit{Astn1} were: 5’ –GCCATGGACCTCTGTGCCCG- 3’; 5’-
CTGAGGGGCAGCCACATGC-3’. 18s rRNA was used as an internal standard for
normalization. Primers for mature microRNA were purchased from Applied Biosystems as: hsa-miR-488 and mmu-miR-488-3p.

**Immunohistochemistry analyses of kidneys** Kidneys collected from 7-8 week old rats, fed either the 0.4% or 8.0% NaCl diet 7 days were fixed in 10% formalin and embedded in paraffin. Sections were cut at a thickness of 5 μm. Slides were deparaffinized in xylene washes and rehydrated with graded series of ethanol. Antigen retrieval was performed with 1X Trilogy (Cell Marque) at sub-boiling temperature for 10 min. Sections were then incubated in PBS with 3% H2O2 for 10 min to inactivate endogenous peroxidase. Slides were washed 5 min in PBST (PBS + 1% Tween 20) and blocked with 3% bovine serum albumin in PBST (blocking buffer) for 2 h at 4°C. Goat anti-Pappa2 or anti-Pappa primary antibody (R&D Systems, 1:1000) was diluted in blocking buffer and incubated at 4°C overnight. Slides were washed three times for 30 min in PBST and incubated in donkey anti-goat HRP secondary antibody as 1:1000 in blocking buffer for 1 hour at RT. Tyramide amplification was performed following the manufacturer’s instructions (Perkin Elmer). Slides were washed 3 × 10 min in PBST (PBS + 1% Tween 20) and blocked with 3% bovine serum albumin for 2 hr at 4°C. Rabbit anti-Aquaporin2 (Santa Cruz, 1:500) or rabbit anti-Na-K-Cl cotransporter2 (ADI, 1:100) or rabbit anti-Na-Cl cotransporter (Millipore, 1:500) primary antibody was diluted in blocking buffer and incubated at 4°C overnight. Slides were washed three times for 30 min in PBST and incubated in goat Alexa-555 coupled secondary antibody (Life technologies) as 1:1000 in blocking buffer for 1 hr at room temperature. Slides were washed three times for 30 min in PBST and mounted with Vectashield containing 4′, 6-
diamidino-2-phenyldole (DAPI). For control with no primary antibody, the tissues were incubated with blocking buffer without the primary antibody included. Similarly for absorption control, the antigen recombinant human Pappa2 (R&D Systems) to anti-Pappa2 was made at a working dilution of 10:1 (molar ratio) and was pre-incubated overnight at 4°C.

Once processed and prepared for imaging, kidney slides were viewed and images captured with Nikon Ni-E Motorized Upright microscope equipped with DS-QiMc camera, NIS-Element software and Nikon ECLIPSE 80i microscope equipped with a QIClick CCD camera (Q imaging) and MetaVue research imaging system (Molecular Devices, CA, USA). All slides were coded such that the observer was blinded to the rat strains and dietary intake.

**Statistical analysis** Data are presented as means ± 1 standard error of the mean (sem). The significance of differences in mean values between and within groups was evaluated using a two way ANOVA for repeated measures for the BP and proteinuria data followed by a Holm-Sidak test for pre-planned comparisons. A two way ANOVA was used for qRT-PCR mRNA comparisons followed by a Holm-Sidak test. For all tests, a $p < 0.05$ using a two-tailed test was considered statistically significant.

**Results**

**Blood pressure and albuminuria before and following 14 days of 8.0% NaCl diet**

Summarized in Table 1 are the blood pressures (mean, systolic, and diastolic blood pressure), heart rate, and urinary albumin excretion data measured on the last day of
0.4% NaCl diet and on day 14 of the 8.0% NaCl diet in SS rats and each of the six overlapping subcongenic strains (26-N, 26-O, 26-P, 26-Q, 26-R, 26-S). There were no statistically differences between any of the strains when maintained on 0.4% NaCl diet for blood pressure or urine albumin excretion. **Figure 1** relates the mean arterial pressure (MAP) and the albuminuria on the last day of the high salt diet (day 14) to the corresponding congenic regions of chr 13 containing BN alleles. The 26-N strain (81.01 to 84.58 Mbp) was protected from salt-induced hypertension and contained the following previously found candidate genes\(^6\) including a portion of *Astn1* and *miR-488, Pappa2, Rfwd2* and *Tnr*. The strains containing *Rfwd2* and *Tnr* BN alleles (26-Q), spanning 81.87 to 84.51 Mbp and 26-S (81.997-82.822 Mbp), were not protected from salt-induced hypertension. Salt-sensitivity was significantly reduced \((p<0.05)\) in four of the subcongenic strains (26-N, 26-O, 26-P, and 26-R) compared to SS rats.

The strains best protected from hypertension salt-sensitivity (26-N, 26-O and 26-P) exhibited significantly lower levels of albuminuria (UNaV) than the SS as shown in **Table 1** and **Figure 1**. Each of these strains contained the BN allele of *Pappa2*, although it cannot be determined if the protection from renal injury was a consequence of the lower levels of hypertension in these strains or independently determined. The 26-R strain, though containing the SS allele of *Pappa2*, did not have a significantly different albumin level compared to the SS even though the MAP was significantly reduced. A small but significant \((P<0.05)\) reduction of heart rate was observed in 26-N, 26-P, and 26-R compared to SS rats.

Among these data, the most notable finding is that the 26-P strain spanning the 81.01 to 81.72 Mbp region which was well-protected from both salt-induced
hypertension and renal injury, contains only three genes, *Astn1, miR-488 and Pappa2*. One or more of these genes or elements within this region, or interactions among them, clearly provided robust protection from salt-induced hypertension. It is these two protein-coding genes and this microRNA that are the focus of the remainder of this report. **Figure 2** compares the progressive changes in MAP before and following changing from the 0.4% to 8.0% NaCl diet in SS and strain 26-P where these three genes are found. Furthermore, we evaluated the 24 hr MAP to determine if the change (delta) in the day time to night time blood pressures was different between the two strains as a means of evaluating whether there was a different circadian pattern. Delta values were generated by calculating the absolute difference between adjacent 12 hr. bins which were then normalized by the earlier of the adjacent time points to account for the effect of pressure itself on the amplitude of the cycles. We performed a 2-way repeated measures ANOVA on the 12-hour-binned normalized ΔMAP data from the SS and 26-P rats. When considered independently of time, normalized ΔMAP did not differ significantly between strains (p=0.555), but did differ significantly over the time course of high salt (p<0.001), indicating an increase in normalized ΔMAP over the 14 days of high salt in both strains. Further, a significant interaction effect between salt and strain was found (p<0.001), indicating that the magnitude of normalized ΔMAP change differed between the two strains. Specifically, 26-P had greater normalized ΔMAP values than the SS on days 10, 12 and 14 (p<0.05).

It should be mentioned that the pre-surgical weight of the SS and the 26-P rats were not different (179±12 and 172±7 gm respectively). At the end of the study
following 14 days of 8% NaCl diet, the weights were still not different between strains (282±21 and 292±5 gm).

**RNA expression in kidneys**  The expression levels of the two protein-coding candidate genes (*Pappa2, Astn1*) and the micro-RNA (*miR-488*) in the renal cortical and medullary tissue were compared in SS and the salt-resistant 26-P congenic strain fed either the 0.4% NaCl diet from weaning (n=6 per strain) or 8.0% NaCl diet for seven days (n=6 per strain). Shown in Figure 3, as determined by qRT-PCR, *Pappa2* mRNA expression was > 8 times higher levels in the renal cortex of the 26-P strain relative to SS rats fed 0.4% NaCl diet. Interestingly, this differential expression was seen only in 26-P rats receiving the 0.4% NaCl diet indicating that *Pappa2* expression levels were influenced by salt diet in a strain-dependent manner. In 26-P rats fed 8.0% NaCl diet for seven days, renal cortical *Pappa2* was reduced to levels not significantly different than SS rats fed the same diet. Cortical *Pappa2* mRNA levels of the SS rats remained low with high salt feeding. *Pappa2* mRNA was also expressed in the outer medullary tissue but at levels too low to make quantitatively reliable comparisons between the strains. *Astin1* mRNA expression did not differ significantly in the renal cortex between SS or 26-P rats fed 0.4% or 8.0% NaCl chow (Figure 3) and was also below quantifiable levels in the renal medulla. Similarly, expression levels of two mature microRNA transcripts, (*miR-488* and *miR-488-3p*) were assessed but only *miR-488-3p* was detected at very low levels in the renal cortex and did not differ between the SS and 26-P rats at either level of salt intake (Figure 3).
To determine if reduced salt-sensitivity was consistently associated with the expression of the renal cortical BN Pappa2 allele, mRNA expression levels were compared by qRT-PCR between six salt-resistant congenic strains containing the BN Pappa2 allele (26-P, 26-J\textsuperscript{6}, 26-F\textsuperscript{6}, 26-M\textsuperscript{6}, 26-N, 26-O) and the four salt-sensitive strains containing the SS Pappa2 allele (SS, 26-Q, 26-R and 26-S). As summarized in Figure 4, Pappa2 was expressed at 6-10 times greater levels in every one of the salt-resistant congenic strains containing the BN allele compared to those strains containing the Pappa2 SS allele. Moreover, when the rat congenic strains containing the BN allele (26-F, 26-J, 26-M, 26-N, 26-O, and 26-P rats) were fed the 8.0% NaCl diet, Pappa2 gene expression was reduced to levels similar to those strains expressing the SS allele (SS, 26-Q, 26-S). It should be noted that gene expression was analyzed in tissue collected after only seven days of high salt to minimize expression differences that might arise as a consequence of prolonged hypertension and renal injury known to be present with the longer period (14 days) of high salt feeding represented in the blood pressure and urine protein data of Figure 1.

**RNA expression in brain stem nuclei and adrenal gland and other tissues** RNA expression of Pappa2, Astn1 and miR-488 (488 and 488-3p) was determined in three brain nuclei (NTS, RVLM, CVLM) to compare expression in SS and 26-P rats fed the 0.4% NaCl diet (n=6-7 rats per strain) and in rats fed 8.0% NaCl diet for seven days (n=6-9 rats per strain). As summarized in Table 2, Pappa2 was expressed at relatively low levels in each of these brain nuclei (1.5 to 7 mRNA copy/10\textsuperscript{6} 18s). No significant differences of Pappa2 expression were found between rat strains fed either 0.4% or 8.0% NaCl diet. Astn1 was expressed at basal levels ranging from 150 to 230 mRNA
copy/10^6 18s, but also exhibited neither significant strain differences nor dietary salt effects. *miR-488-3p* mRNA was expressed at low levels in all of the selected brain tissues (0.06 to 0.23 mRNA copy/10^6 5s). Within this context, no significant differences in *miR-488-3p* were observed in the NTS between SS and 26-P rats, nor were expression levels affected by salt diet. Within the CVLM, *miR-488-3p* expression levels were higher in SS rats compared to 26-P rats (N= 5-7; p<0.05) fed the 0.4% NaCl diet but the high salt diet did not significantly change these levels in either strain. Within the RVLM, expression of *miR-488-3p* was higher (p<0.05) in SS rats receiving the 8.0% NaCl diet compared to 26-P rats but salt diet did not result in significant changes in either strain. Similarly, as also shown in Table 2, no significant differences were observed in the adrenal gland tissue between the SS and 26-P rats at either level of salt. Finally, in a screen of the SS and 26-P strains (one rat of each strain and each salt level) of left ventricle, thoracic aorta, abdominal muscle and liver, no salt or strain differences were suggested and so subsequent analyses were not performed.

Taken together, the results indicate that of the three genes, only *Pappa2* mRNA was differentially expressed in the renal cortex between the SS and 26-P rats and the only gene of the three modified by salt diet in this organ.

**Genome sequence analysis in the 26-P congeneric interval** The genetic analysis of our data indicates that causative variants reside in the 0.71 Mbp interval of the 26-P region. *Pappa2* was not annotated in rat genome assembly Rn5 but there were two LOC genes (LOC 680069 and LOC680415) predicted in Rn4 in this region. LOC680415 was found to be part of the gene from LOC68069 if the orientation was reversed. The rat *Pappa2* gene cDNA sequence was derived by aligning RNA-seq
transcript, the predicted LOC gene sequences, and the EST sequences starting at the first ATG. When the synthesized Pappa2 gene was transfected into bacteria, the overexpressed protein could be detected by an anti-Pappa2 antibody with the anticipated molecular weight of 250 kD (data not shown).

Based on this reassembly, the narrow 0.71 Mbp interval contains 1097 SNPs and 436 Indel variants comparing SS/JrHsdMcwi and BN/NHsdMcwi. Six SNPs reside in the Pappa2 gene (cDNA) and one of these was predicted by Polyphen-2 to be a benign nonsynonymous change (T1340A). There were seven nonsynonymous SNPs found in the Astn1 cDNA, and three SNPs in the 3' UTR region. In addition, 436 insertions/deletions (Indels) reside within the 710-kb region, most of them are in introns and only four synonymous changes were found in Astn1 gene. There are eight SNPs, five insertions and two deletions among the sequences 25 kb upstream from the translation start codon of Pappa2 (81,574,056-81,600,000 site). It is possible these could cause differential expression of Pappa2 in the kidney cortex. However, since Pappa2 exhibits cell type specificity, in silico analysis (such as TRANSFAC) could not be applied, the functional relevance of these mutations remains to be experimentally determined.

It should be noted that we have used the Rn5 for our assembly since Rn6 did not provide any additional useful information in the candidate region. Analysis of the genome alignment from Ensembl and UCSC Genome Browser in general showed a very similar alignment in both browsers. Yet, we found no alignment of Pappa2 in the UCSC Genome Browser RGSC Rnor_6.0/rn6. Astn1 was determined as known processed transcripts instead of a protein coding gene in the Rat Genome Database
(rgd.mcw.edu) indicating incompleteness of the transcripts in Ensembl Rnor_6.0. Using the information of genetic variants presented in Ensembl Rnor_6.0, Pappa2 showed three meaningful variants as were found in Rn5. One SNP was additionally found in our sequence comparison of SS and BN (T1340A).

**Localization of Pappa2 in renal cortex and medulla**  
Figure 5 shows a representative photomicrograph of the immunohistochemical characterization of Pappa2 protein expression in the kidney. Kidneys collected from the same group of seven week old rats used in the gene expression analysis were utilized for these analyses (N=6/salt diet, SS rats and N=6/salt diet, 26-P rats). Salt-resistant 26-P rats receiving the 0.4% NaCl diet (top right panel; 4X magnification) typically exhibited expression of Pappa2 largely in the renal cortex and to a lesser extent in the medulla. In contrast, SS rats fed the same salt diet exhibited little Pappa2 in either the cortex or medulla (top left panel; 4X magnification). Pappa2 immunofluorescence was greatly reduced in 26-P rats when fed the 8.0% NaCl diet (lower right panel; 4X) with immunofluorescence levels similar to those of SS rats (lower left panel; 4X). Consistent with the Pappa2 mRNA qRT-PCR data, these higher levels of Pappa2 protein immunofluorescence were observed in each of the six 26-P rats compared to six SS rats fed 0.4% NaCl diet.

Primary antibody staining and absorption control for the Pappa1 protein was carried out to assess possible cross-reactivity between Pappa2 and Pappa1. Pappa1 immunofluorescence was found to be localized predominately in the renal medulla equally in both SS and 26-P rats (fed the 0.4% NaCl diet) as distinct from the Pappa2 which was observed largely in the cortex of the 26-P rats.
A more precise localization of Pappa2 was obtained by immunocolocalization studies at 40X magnification of the tissue sections from SS and 26-P rats fed the 0.4% NaCl diet. Figure 6 (40X) shows co-staining of the Na⁺K⁺Cl⁻ cotransporter (NKCC2) fluorescence antibody (marker of tubular thick ascending limbs) with that of Pappa2 in cortical thick ascending limbs (cTAL) but not the medullary thick ascending limbs (mTAL) of 26-P rats fed the 0.4% NaCl diet.

Also shown in Figure 6 is the co-staining of Pappa2 with Aquaporin 2 (Aqp2), the marker of cortical and medullary collecting ducts. As illustrated in these images, Pappa2 was colocalized with Aqp2 in both the cortex and medullary collecting ducts extending to the tip of the papilla (not shown). However, Pappa2 was not colocalized to the same cells that expressed the Aqp2 (e.g., principal cells) indicating that Pappa2 is uniquely bound to the apical membranes of the intercalated cells.

Pappa2 was also intermittently observed within the tubular lumen and associated with apical membranes of the epithelial cells, but was not apparent intracellularly as demonstrated by co-expression with the fluorescent antibody for the distal tubular marker, Na⁺Cl⁻ cotransporter (NCC) as shown in Figure 7. The co-expression of Pappa2 and NCC was observed in both SS and 26-P rats but most conspicuously in the cortex of the 26-P rats fed the 0.4% NaCl diet. As discussed below, this observation is consistent with secretion from more proximal segments such as the cTAL.

**Discussion**

The goal of the present study was to fine map genes relevant to BP salt-sensitivity within chr 13 of the SS rat. Previous exclusion mapping localized a 1.37 Mb segment of chr 13 containing five genes (Fam5b, Astn1, Pappa2, Rfwd2 and Tnr)⁶. In
the present study, this region was further narrowed and captured within an inbred conegenic strain to 0.71 Mbp, which contains BN alleles of two genes (Astn1 and Pappa2), and a microRNA (miR-488-3p). This conegenic strain (26-P) exhibited significant protection from salt-induced hypertension. This 26-P conegenic region excludes the Rfwd2, Fam5b and Tnr genes, one of which, Rfwd2, we have previously suggested could be important in determining blood pressure diurnal rhythms⁶.

The novel result of the present study is that among the three genes within the 0.71 Mbp conegenic 26-P region, only Pappa2 mRNA and immunofluorescence of Pappa2 protein binding was greater in the renal cortex of 26-P compared to the SS rat on the 0.4% NaCl diet. This differential expression was uniquely observed only in rats fed the lower 0.4% NaCl diet and not in those fed the high 8.0% NaCl diet. Remarkably, differential mRNA expression of Pappa2 in the renal cortex was observed in every one of six overlapping salt-resistant conegenic strains of rats containing the BN Pappa2 allele when compared to SS rats as illustrated in Figure 4. In all cases, Pappa2 mRNA expression levels were reduced when these conegenic strains were fed 8.0% NaCl diet. Importantly, several lines of evidence indicate that Pappa2 is the gene most likely responsible for the protection from salt-sensitivity in this region of chr 13. First, neither Pappa2 nor Astn1 expression differed between the SS and 26-P rats within the non-renal tissues that were analyzed. Second, Pappa2 was the only one of the three genes within the renal cortex that was significantly different between the SS and 26-P rats. Third, Pappa2 was the only one of the three genes that responded consistently in the 26-P rats to changes of salt diet.
Not unexpectedly, as the many overlapping congenic strains were analyzed, there was some evidence of gene-gene interactions, as exemplified by strain 26-R. From Figure 3 it can be seen that 26-R *Pappa2* expression levels were more in line with those expected from an SS allele, yet the MAP of the 26-R strain was clearly reduced (144 ± 8 mmHg) compared to the SS rats (163 ± 16 mmHg) as shown in Figure 1. This suggests the existence of genetic elements that can modulate BP in this congenic interval, although it is difficult to conjecture which of the 32 genes contained in this region could be responsible for modulating the BP. Yet, some of these genes have interesting functions, such as DARS2 (mitochondrial aspartyl-tRNA synthetase), which is required for normal mitochondrial protein synthesis in cardiomyocytes\(^{10}\) and others that are involved in the TNF signaling pathway that could affect BP.

*Pappa1 and Pappa2* are metalloproteinases in the metzincin superfamily. Pappalysin-1, also known as pregnancy-associated plasma protein, is encoded by the *Pappa* gene. *Pappa1* in the kidney appears to be largely expressed in the renal medulla in a similar manner in both the SS and 26-P rats fed 0.4% NaCl diet. It is, therefore, not colocalized with *Pappa2* in the kidney which, as shown in Figure 4, is largely evident in the renal cortex. Both *Pappa1* and *Pappa2* genes have been reported to be found in the plasma, kidney, uterus, adipose tissues, heart and placenta\(^5,19\). It is of interest that *Pappa1* is a secreted protein, found in the circulation, and has been analyzed by immunoassay under a variety of pregnancy related conditions\(^{22,29}\). *Pappa2* has not been extensively studied, although it is recognized to be a protease which can cleave IGFBP-5\(^{42,30}\), thereby regulating hydroxyapatite and IGF-1 binding\(^4\). However, IGFBP-
is also able to exert biological activities independent of IGF-1\textsuperscript{34}. Although clinical associations between Pappa2 and IGF-1 and hypertension and cardiovascular risk have been reported\textsuperscript{19,35,41}, the mechanistic link is unclear. Knock out of \textit{Pappa2} in mice was observed to result in post-natal growth retardation\textsuperscript{5}, although kidney weights, blood pressure and cardiovascular parameters were not determined so the relevance of these observations to hypertension is unclear.

**Localization of Pappa2 in the rat kidney** As identified by immunohistochemistry, \textit{Pappa2} was effectively localized in the kidneys of congenic 26-P rats fed the 0.4\% NaCl diet (Figure 5) where mRNA expression levels were found to be significantly higher compared to SS rats (Figure 4). The fluorescent signal of the Pappa2 protein was consistently greater in the renal cortex compared to the renal medulla as seen in the 4X images of the hemisected whole kidney. Moreover, as seen in the 40X images (Figure 6), Pappa2 in the cortex was specifically colocalized with NKCC2 near the glomerulus and was observed within the cytosol of the cTAL epithelial cells of this region. These were the only tubular segments in which the Pappa2 protein appeared to be expressed intracellularly. Pappa2 was also colocalized intermittently with NCC expressing cells of the distal tubules at the apical membranes (Figure 7). Most notably, Pappa2 consistently colocalized with Aqp2 positive expressing tubular segments of the cortex and outer medulla. Of particular relevance to future studies, Pappa2 in the collecting ducts did not colocalize to the Aqp2 expressing principal cells, but specifically to the apical membranes of the intercalated cells, which did not express the green Aqp2 fluorescence.
Co-Regulation Database analysis (CORD)  It is challenging to construct a functional hypothesis linking Pappa2 to renal function and BP regulation at this time. Functionally, there exist only fragments of information from which one could deduce the relevance of the cortical responses of Pappa2 in the congenic 26-P rats to increased intake of dietary salt as reported in the present studies. To infer possible functional roles of Pappa2, we carried out a meta-analysis of gene expression databases using the Co-Regulation Database\(^{13}\) (CORD; \(\text{http://cord-db.org}\)) to identify genes that commonly co-express with Pappa2. CORD queries more than 120,000 array datasets to identify genes that express either positive or negative correlation with a user-specified target gene. When analyzing the genes that correlate with Pappa2, 400 genes were found with an absolute Pearson’s correlation coefficient of over 0.35 and seven genes had a correlation coefficient over 0.75. These most highly correlated genes included vascular endothelial growth factor receptor 1 (\(\text{FLT1}; r = 0.825\)); placenta-specific 1 (\(\text{PLAC1}; r = 0.809\)); disrupted in renal carcinoma 2 (\(\text{DIRC2}; r = 0.791\)); tissue factor pathway inhibitor 2 (\(\text{TFPI2}; r = 0.785\)); ceroid-lipofuscinosis neuronal 3 (\(\text{CLN3}; r = 0.768\)); amiloride binding protein 1 (\(\text{ABP}; r = 0.767\)) and stimulated by retinoic acid gene 6 homolog (\(\text{STRA6}; r = 0.755\)). Six other genes associated with Na+ transport showed a high correlation with Pappa2 expression (correlation coefficient > 0.75) including \(\text{Slc12a2}, \text{Slc13a4, Slc23a2, Slc5a6, Scn4b, Scnn1b}\). Five genes also showed high correlation with regulation of blood pressure including \(\text{Acvrl1}, \text{Adipoq, Hsd11b2, Nos3, and P2RX4}\). However, since most of the microarrays queried by CORD were neither renal or vascular, the pathways themselves were not statistically overrepresented and it is
evident that the observed gene correlations are only suggestive of some possible mechanisms whereby Pappa2 could be influencing blood pressure. Together, these associations broadly suggest that Pappa2 is associated with genes involved in tubular sodium transport, cell growth and differentiation. Since Pappa2 cleaves IGFBP-5 releasing IGF, enhancement of downstream signaling of mTORC1 with stimulation of the cell cycle pathway would be expected\textsuperscript{17}.

To identify genes that were coregulated with both Pappa2 and IGFBP-5, another CORD analysis was carried out. Although the majority of genes that were co-regulated with Pappa2 were not also co-regulated with IGFBP-5, (and vice-versa), 16 genes were identified that were together co-regulated. These included: Adam 12, CKDN1C, CD200, COL4A1, Col4A2, FBLN1, FBN2, MFAP2, PLAGL1, PPAP2B, SERPINE2, TIMP2, GEMIN6, LYAR, MTHFD1 and PRPS1. An ontology analysis performed on this list of 16 using DAVID (Database for Annotation, Visualization and Integrated Discovery), yielded an average enrichment score of 4.24 with these genes found to be most related to extracellular matrix and disulfide bond pathways. Taken together these two CORD analyses indicate that Pappa2 has roles related to both Na\textsuperscript{+} transport functions and the IGF system, either coordinately or independently.

**Possible mechanisms** The present study indicates that Pappa2 represents a novel gene which is involved in BP salt-sensitivity in the SS rat. Pappa2 was found to be regulated within the renal cortex of those congenic rats containing the BP Pappa2 allele and was expressed at significantly greater levels in those salt-resistant congenic rats than in the SS rats when fed a relatively low salt diet. Although reduction of renal Pappa2 in response to a high salt diet in the salt-resistant rats could serve a sodium
homeostatic function in those strains with the BN Pappa2 allele, such a mechanism could even be an advantage in the SS rat where Pappa2 expression is already very suppressed even when fed a low NaCl diet. One could speculate that the low levels of Pappa2 expressed in the cTALs of the SS may serve a counterregulatory function that would reduce nephron sodium absorption to partially offset excess sodium absorption known to occur in the thick ascending limbs\cite{21,33}. It would be informative but a large task to determine the sequential changes of the cortical Pappa2 levels after switching to the high salt diet.

It is interesting to speculate how the production of Pappa2 in cTAL could affect BP salt-sensitivity. The cytosolic presence of Pappa2 in the cTAL close to the glomerulus suggests that it could serve, via the macula densa, to modify the gain or set point of the tubular glomerular feedback mechanism. The luminal presence of Pappa2 in more distal tubular segments with localization to only the apical membranes of the downstream tubular segments suggest it may be secreted from the TAL and act downstream to modify tubular function. In a preliminary Western blot analysis of urine, Pappa2 was clearly present. Secretion of Pappa1 is well established\cite{22,29} and it is reasonable to expect the same is true for Pappa2. Given the specific localization of Pappa2 to the intercalated cells of the collecting ducts, it could be involved in the regulation of Na⁺ excretion via alterations of luminal tubular H⁺ or HCO₃⁻ secretion. This would be consistent with evidence that elevations of luminal HCO₃⁻ concentration modulate ENaC abundance and function in principal cells which enhance net NaCl reabsorption\cite{39}. Also, given the results of the \textit{in silico} CORD analysis indicating high
concordance of *Pappa2* with both IGF and Na$^+$ transport functions, it is possible that *Pappa2* could affect Na$^+$ homeostasis and BP salt-sensitivity through these pathways.

The CORD analysis also suggests that *Pappa2* could exert systemic vascular actions. Vascular endothelial cells from both large and microvessels express both IGF1 and the IGF Binding Protein 5 (IGFBP5)$^3$ which as indicated above can be cleaved by Pappa2$^{30,42}$ thereby determining the bioavailability of IGF1 to its receptor binding site. IGF1 receptor activation signals downstream pathways including Ras/Raf/ERK and PI3 kinase/AKT thereby modify vascular function$^2$. So it is possible that as tissue or circulating levels of Pappa2 are reduced, systemic vascular function could be modified as less IGFBP5 is cleaved thereby reducing the bioavailability and vascular effects of IGF$^{37}$. Several studies have suggested that low-normal serum IGF1 levels correlate with an increased risk of adverse cardiovascular outcomes, including myocardial infarction and heart failure$^{12,16}$. Although no studies are yet available examining either the presence or function of either Pappa2 or possible IGFBP5/IGF pathways in the kidney, we have preliminary Western blot data indicating that the renal cortex expresses high IGFBP5 so it appears that at least the elements for such a hypothesis exist in the kidney.

The unbiased search for candidate genes responsible for salt-sensitive hypertension via techniques of chromosomal substitution is a long and expensive process, but a rewarding one, since it can yield unexpected and novel results. Such was the case in the present study, since one would not have guessed that *Pappa2* would be revealed as a viable candidate gene contributing to salt-sensitivity in this long-studied Dahl S rat model of hypertension. *Pappa2* has also never been recognized to
reside in the cortical thick ascending limbs of Henle. Nor has it been known to be
associated with intercalated cells of renal collecting ducts, much less suggested as a
gene regulated by dietary salt intake with a possible role in tubular sodium reabsorption.
These observations now point to the need to examine whether the knockout of Pappa2
in salt-protected congenic rats will also alter sodium transport in collecting ducts and
affect sodium homeostasis leading to salt-sensitive hypertension.
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Disclosures/Conflicts of interest - none


**Figure Legends**

**Figure 1:** Schematic representation of the 3.62 Mbp region of chr 13 spanned by six overlapping congenic strains. Various lengths of chr 13 from the Brown Norway rat (indicated by black bars) were introgressed into the genetic background of the SS rat (white bar). Mean arterial pressure (MAP) measured on day 14 of 8.0% NaCl diet is listed below each strain along with urinary excretion of albumin (UalbV) for the same day of high salt diet. Number of animals is listed below each strain (N). * indicates significant differences from the SS strain on day 14 HS ($p<0.05$).

**Figure 2:** Mean arterial pressure (MAP) was measured on 0.4% NaCl diet for three days and for 14 days after the diet was switched to 8.0% NaCl diet in SS (closed circles; n=11) and 26-P (open circles; n=14). † indicates significant difference between SS and 26-P ($p<0.05$); * indicates significant difference of a 8.0% NaCl diet day from the three control days on 0.4% NaCl diet within a strain ($p<0.05$).

**Figure 3:** mRNA expression of *Pappa2, Astn1* and *miR-488-3p* in renal cortex homogenates collected from SS rats and congenic 26-P rats fed either 0.4% NaCl diet (hatched bars; n=6/strain) or seven days of 8% NaCl diet (black bars; n=6/strain). mRNA copy number was normalized to 18S. The microRNA expression of *miR-488-3p* in the renal cortex from same animals was normalized to RUN6B. * indicates significant difference between strains on the same salt diet ($p<0.05$).

**Figure 4:** mRNA expression of *Pappa2* in homogenate of renal cortex collected from six subcongenic strains containing the Brown Norway (BN) allele (26-F, 26-J, 26-M, 26-N, 26-O, 26-P) and four strains containing the SS allele for *Pappa2* (SS, 26-Q, 26-R,
26-S) fed either 0.4% NaCl (hatched bars; n=6/strain) or seven days of 8% NaCl diet (black bars; n=6/strain). Note that blood pressure phenotypes of 26-F, 26-J and 26-M were previously reported and that tissue was not available from the 26-R strain fed 8% NaCl for seven days for mRNA analysis. mRNA levels of Pappa2 were normalized to the expression level in the SS rat on 0.4% NaCl diet. *p<0.05 compared to 0.4% NaCl diet within strains; †p<0.05 compared to SS fed 0.4% NaCl diet.

**Figure 5:** Representative photomicrograph of Pappa2 immunofluorescence in the renal cortex and outer medulla of seven week old (7wk) SS and subcongenic 26-P rats fed 0.4% NaCl diet (top panel; 4x magnification) and 8% salt (lower panel; 4x magnification). Superimposed white lines identify the boundaries of the cortex (C) and medulla (M). As shown, Pappa2 expression was higher in the renal cortex of 26-P rats fed the 0.4% NaCl diet (right upper panel) compared to SS rats. The fluorescence signal was greater in the cortex compared to the medulla. It is also seen that Pappa2 fluorescence levels were lower in 26-P rats fed the high 8.0% NaCl diet (right lower panel) compared to the rat fed the 0.4% NaCl diet (right upper panel). SS exhibited relatively low levels of Pappa2 fluorescence in both regions from rats on both salt diets (left upper and lower panels).

**Figure 6:** The top panel illustrates the co-localization by immunohistochemical methods of Pappa2 (red fluorescence) and the Na⁺K⁺Cl⁻ cotransporter 2 (NKCC2) in renal cortex and medulla (40x magnification) and the lower panel the co-localization of Pappa2 (red fluorescence) with aquaporin 2 (Aqp2; green fluorescence) in the two regions. Pappa2 was colocalized with NKCC2 and appears to reside in the cytosol of the cortical thick ascending limbs of this region (cTAL; seen as the yellow/orange...
fluorescence color) as found in the seven week old 26-P rats (26-P 7W) fed the 0.4% NaCl diet. Note that Pappa2 appears to be expressed intracellularly. There is not a co-localization with the Aqp2 expressing principal cells (green) but rather in other cell types which appear to be intercalated cells (red).

**Figure 7:** Pappa2 is shown co-expressed with the thiazide-sensitive Na\(^+\)-Cl\(^-\) cotransporter (NCC) fluorescence antibody (distal tubular marker) in the cortex of only the 26-P rats fed 0.4% NaCl (right panel) and not in SS rats (left panel). Note that Pappa2 (red) is localized to the apical membrane of distal tubular epithelial cells (right panel) and not intracellularly. Pappa2 (red) can also be seen within the tubular lumen of the distal tubules in both the SS and 26-P rats (left and right panels).
Table 1: Blood pressure and urine albumin excretion measured on 0.4% NaCl diet and after 14 days of 8.0% NaCl diet. Mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR) were measured from 9 a.m. to 12 p.m. Urine albumin excretion (UalbV) was determined after an overnight collection.

<table>
<thead>
<tr>
<th>0.4% NaCl</th>
<th>SS</th>
<th>26-N</th>
<th>26-O</th>
<th>26-P</th>
<th>26-Q</th>
<th>S6-R</th>
<th>26-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP (mmHg)</td>
<td>107±1</td>
<td>105±2</td>
<td>103±2</td>
<td>107±1</td>
<td>107±1</td>
<td>105±2</td>
<td>106±1</td>
</tr>
<tr>
<td>SBP</td>
<td>122±1</td>
<td>117±2</td>
<td>117±2</td>
<td>122±2</td>
<td>121±1</td>
<td>119±1</td>
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<td>DBP</td>
<td>88±1</td>
<td>90±2</td>
<td>86±2</td>
<td>89±2</td>
<td>89±1</td>
<td>86±2</td>
<td>88±2</td>
</tr>
<tr>
<td>HR</td>
<td>414±5</td>
<td>401±4</td>
<td>395±5</td>
<td>397±4</td>
<td>413±5</td>
<td>402±7</td>
<td>405±5</td>
</tr>
<tr>
<td>UalbV</td>
<td>2.8±0.5</td>
<td>1.7±0.4</td>
<td>1.2±0.3</td>
<td>2.2±0.3</td>
<td>2.9±0.5</td>
<td>4.1±1.2</td>
<td>2.6±0.6</td>
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<table>
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<th>8.0% NaCl 14 days</th>
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<tr>
<td>MAP (mmHg)</td>
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<tr>
<td>SBP</td>
</tr>
<tr>
<td>DBP</td>
</tr>
<tr>
<td>HR</td>
</tr>
<tr>
<td>UalbV</td>
</tr>
</tbody>
</table>

N= 14 15 11 11 13 10 15

Values given are mean ± sem; number of rats N=; * indicates significant difference between strains on the same salt diet (p<0.05)
Table 2: RNA expression of *Pappa2*, *Astn1* and *Mir-488-3p* in three specific brain regions and the adrenal gland collected from SS rats and congenic 26-P rats fed either 0.4% or switched to 8.0% NaCl diet for 7 days (7D).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Pappa2 mRNA (copy number/10⁶ 18s)</th>
<th>Astn1 mRNA (copy number/10⁶ 18s)</th>
<th>mir-488-3p (copy number/10⁵ 5s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SS 0.4%</td>
<td>SS 8.0 % 7D</td>
<td>SS 0.4%</td>
</tr>
<tr>
<td>brain CVLM</td>
<td>3.69±1.05(5)</td>
<td>7.30±3.7(7)</td>
<td>4.51±1.92(5)</td>
</tr>
<tr>
<td>brain RVLM</td>
<td>2.79±0.82(6)</td>
<td>3.04±1.59(7)</td>
<td>4.16±2.30(7)</td>
</tr>
<tr>
<td>brain NTS</td>
<td>1.40±0.17(7)</td>
<td>1.68±0.26(7)</td>
<td>1.58±0.44(6)</td>
</tr>
<tr>
<td>adrenal gland</td>
<td>0.06±0.01(5)</td>
<td>0.08±0.21(5)</td>
<td>0.11±0.03(6)</td>
</tr>
</tbody>
</table>

CVLM=caudal ventrolateral medulla; RVLM=rostral ventrolateral medulla; NTS=nucleus tractus solitaries; values given are mean ± sem; number of rats indicated in parentheses; * indicates significant difference between strains on the same salt diet (p<0.05)
MAP (mmHg) vs. Day for 8% NaCl diet
**Pappa2**
renal cortex

mRNA copy/10^8 18s

SS | 26-P
---|---

*P* > 0.05

**Atn1**
renal cortex

mRNA copy/10^8 18s

SS | 26-P
---|---

**miR-488-3p**
renal cortex

copy/10^4 RUN6B

SS | 26-P
---|---
0.4% NaCl diet
SS renal cortex  26-P renal cortex

0.4% NaCl diet