Analysis of the Role of the SA Gene in Blood Pressure Regulation by Gene Targeting

Vanessa Walsh, Laurence Somody, Aldo Farrell, Bei Li Zhang, Jane Brown, Catrin Pritchard, Madeleine Vincent, Nilesh J. Samani

Abstract—The SA gene is expressed in the proximal tubule of the kidney and may be involved in blood pressure (BP) regulation. However, direct evidence for this is lacking. We constructed and analyzed an SA-null mouse in which exons 2 and 3 of the SA gene (including the start codon) had been deleted by homologous recombination. Basal BP and BP changes in response to increased salt and to treatment with losartan were compared between mice homozygous for the targeted SA allele (SA−/− mice) and littermates carrying the wild-type allele (SA+/- mice). Molecular and biochemical analysis confirmed the lack of SA gene product in SA−/− mice. SA−/− mice grew normally, were fertile, and had no overt phenotype. With both indirect and direct techniques, basal BP was similar in SA−/− and SA+/- mice. A high salt diet for 4 weeks caused a significant increase in BP in SA−/− and SA+/- mice, but there was no difference between the 2 strains. Losartan caused a significant decrease in BP, but again the response was similar between SA−/− and SA+/- mice, as were their kidney renin mRNA levels. SA is not involved in the regulation of either basal or salt related BP, and the lack of differential effect in SA−/− mice is not a consequence of compensatory activation of the renin-angiotensin system. (Hypertension. 2003;41:1212-1218.)

Key Words: hypertension, genetic ■ kidney ■ genes ■ genetics ■ renin-angiotensin system

The SA gene was identified by differential hybridization because of its much higher expression in the kidney of the spontaneously hypertensive rat (SHR), a genetic model of essential hypertension, compared with the Wistar-Kyoto rat. Although the function of SA was not immediately apparent from its primary structure encoding a protein of 578 amino acids, its increased expression in an organ known to play an important role in the underlying cause of hypertension in the SHR raised the possibility that it may be part of a novel system regulating systemic blood pressure (BP) and contributing to hypertension of the SHR. Linkage of the SA locus on rat chromosome 1 to blood pressure (BP) variation in progeny of recombinant crosses involving the SHR, the coincidence of its increased expression in the SHR to the rapid phase of hypertension development, and the location of its expression to the proximal tubule all supported this possibility. In humans, association studies of polymorphisms in the SA gene with hypertension have reported mixed findings, although a recent study in which a comprehensive evaluation of the gene for polymorphisms was followed by association studies in a large cohort of subjects found significant association of a possibly functional polymorphism in intron 12 with BP and other cardiovascular phenotypes. Despite these data, direct evidence of a role for SA in BP regulation is lacking. In this study, we investigated this by constructing and analyzing an SA-null mouse.

Methods

Preparation of an Embryonic Stem Cell Line With a Targeted SA Gene

The mouse SA gene was isolated on 5 overlapping bacteriophage clones from a 129Sv strain chromosomal DNA library (Stratagene Europe), using a 1.6-kb rat SA cDNA as probe. Detailed restriction enzyme mapping of the clones was carried out, and the location of exons (including exon 3, which contains the start ATG codon) was determined with the use of exon-specific CDNA or oligonucleotide probes, based on the known structure of the rat SA gene. A targeting vector, pVax6, was constructed by cloning 2 fragments of the mouse SA gene into the vector pX53 (Figure 1). The 5′ homology arm consisted of a 3.3-kb Sal I/EcoR I intronic fragment upstream of exon 2, whereas the 3′ homology arm consisted of a 3.2-kb EcoR I/Xba I fragment containing exon 4 and parts of the adjacent introns. Homologous recombination of the targeting vector with the wild-type SA gene was predicted to result in replacement of a 3.3-kb fragment of the SA gene (including exons 2 and 3) with the Neo gene and the creation of a novel Hind III site in the targeted gene (Figure 1); 5×107 embryonic stem (ES) cells from the mouse strain 129/Ola (E14TG2a) were transfected with 60 μg of Not I linearized pVax6. After application of positive (G418) and negative (gancyclovir) selection, ES clones were screened by Southern blot analysis of DNA digested with Hind III for the occurrence of a
**Construction of an SA**⁻**⁺ Mouse**

ES cell clones containing a targeted SA gene were grown and microinjected into F1(C57BL/6J × CBA) blastocysts. Blastocysts were reimplanted into pseudopregnant F1(C57BL/6J × CBA) female mice and progeny assessed by coat color for chimerism. Chimeras were initially bred with MF-1 mice to screen for germline transmission using coat color and those that showed this, subsequently with 129/Sv mice. Progeny were genotyped (see below) to identify carriers of the targeted SA allele and 2 further cycles of back-crossing carried out of SA⁻⁺ animals with 129/Sv animals to produce a partially inbred line (87.5% 129/Sv; 12.5% 129/Ola). The line was subsequently maintained by brother-sister mating of SA⁻⁺ animals and progeny used for experimental studies. All procedures were carried out in accordance with our institutional guidelines.

**SA Genotyping**

Tail DNA was prepared as previously described. For each sample, 2 separate PCRs were performed to establish the SA genotype. In the first reaction, a 95-bp fragment of exon 2 of the SA gene (identifying the positive allele) was amplified using the primers MSA12 (sense 5’-AGATCACCAGACTCAGGAPGT 3’) and MSA17 (antisense 5’-GTTGCTTTCAGCATCATGCTG 3’). In the second reaction, a 504-bp fragment of the neomycin resistance gene (identifying the negative allele) was amplified using the primers OCP49 (sense 5’-GTTTCTCCGCGCCGTTTGGTGGAG 3’) and OCP50 (antisense 5’-GGGCGGGCTTGGACGCGGAGA 3’). By considering the results of both reactions, animals could be classified as SA⁺⁺, SA⁻⁻, or SA⁻⁺.

**RNA Analysis**

Total kidney and liver RNA was prepared from 16-week-old animals. Steady-state mRNA levels for SA, GAPDH (loading control), KS, and renin (kidneys only) were assessed by standard Northern blotting, using as probes a 1.6-kb rat SA cDNA, a 29-mer complementary oligonucleotide to GAPDH, a 361-bp cDNA (nucleotides 1134 to 1495) to the KS gene, and a rat renin cDNA, respectively. All probes were radioactively labeled with 32P-dCTP. Signals were quantified by densitometry (AlphaImager, Alpha Innotech). The presence of SA protein was detected with a polyclonal rabbit SA antibody (SA106) raised against a peptide (N-E-L-R-R-K-E-W-T-T) of the rat SA protein. The secondary antibody was a peroxidase-conjugated, goat anti-rabbit immunoglobulin (Dako, 25 μg/L) used at a dilution of 1:5000 and detected with the use of an enhanced chemiluminescence kit (Amersham Pharmacia Biotech). In peptide block control experiments, SA peptide was added to the primary antibody at a concentration of 5 μg/mL before its incubation with the membrane.

**Phenotype Analysis of SA⁻⁻ Mice**

Effect on fetal survival was assessed by counting the number of progeny of different genotypes obtained from mating of the heterozygote animals (see above). Effect on growth was assessed by comparing the body and organ weights of SA⁺⁺ and SA⁻⁻ mice at 16 weeks. Plasma urea and creatinine were measured with an automated hospital analyzer (Abbott Aeroset Analyser, Abbott Laboratories Ltd). Kidneys of SA⁺⁺ and SA⁻⁻ mice were examined by light microscopy to look for any effects on kidney architecture and by electron microscopy to investigate any effects on the ultrastructure of proximal tubular cells.

**Assessment of Hemodynamic Consequences of Lack of SA**

This was assessed with the use of 2 protocols. In the first study, 30 male SA⁺⁺ mice and 28 male SA⁻⁻ littermates had basal BP measured at 12 weeks (mean of 3 measurements per day on 2 consecutive days for each animal) by tail-cuff plethysmography, using a system (IITC) validated for BP measurement in mice. Half of each group of animals were then randomized to continue with a normal diet and half were placed on a high salt diet (1.5% NaCl in drinking water). BP was measured after 4 weeks on this diet. In the second study, 10 SA⁺⁺ and 10 SA⁻⁻ male mice were implanted with a mouse radiotelemetry probe (Data Sciences International) in the thoracic aorta by means of a carotid approach at 15 weeks of age. Mice were anesthetized by intraperitoneal injection of 0.7 mL/10 g body wt of a mixture of fentanyl-fluanisone, (0.2 mg/mL), midazolam (5 mg/mL), and sterilized water at a volume ratio of 1:1:2. Eight SA⁺⁺ and 8 SA⁻⁻ mice survived the surgery. Probes failed after implantation in 1 SA⁺⁺ and 3 SA⁻⁻ mice. The remaining mice were allowed to

![Diagram](image-url)
recover for 1 week. Basal BP was then measured for 2 weeks. This was done for 2 consecutive, full 24-hour periods at the end of each week (8 AM to 8 AM). Animals were then placed on a high salt diet (1.5% NaCl in drinking water) for 4 weeks. BP was again measured continuously for the last 2 days in each week. Animals were then placed back on a normal diet for 2 weeks (recovery period), and, after a further BP assessment, were treated orally with the angiotensin receptor antagonist losartan (30 mg/kg per 24 hours) for 2 weeks. A final BP was then obtained after a further BP assessment, were treated orally with the angiotensin receptor antagonist losartan (30 mg/kg per 24 hours) for 2 weeks. BP was measured for the first 2 days and at the end of the 1st and 2nd weeks of treatment. A final BP was then obtained after a further recovery period of 2 weeks. BP measurements were made beat-to-beat and stored as 2-minute averages. Mean 24-hour as well as day (8 AM to 8 PM) and night (8 PM to 8 AM) systolic BP (SBP) and diastolic BP (DBP) were calculated across treatment periods, average SBP and DBP were calculated for the 2 weeks of basal readings, the last 2 weeks of the salt period, the final 2 weeks of losartan and the final BP. Statistical Analysis

Quantitative data are shown as mean ± SEM. Values in SA+/+ and SA−/− mice were compared by means of ANOVA. Effects of treatment within each group were compared by paired t test. The independent effects of strain and of treatment and any interaction between these on BP were assessed by general linear modeling. Genotype frequencies were assessed by χ² test. All statistical tests were carried out with the use of Minitab Version 13.0 (Minitab Inc).

Results

Of 294 G418-resistant ES cell clones screened, 11 clones (3.7%) gave a novel HindIII SA fragment arising from the targeted gene. PCR-based genotyping of DNA from progeny of mating of heterozygous animals to identify /−, +/+, and +/+ animals. Upper panel shows genotyping for 95-bp exon 2 product (only present in the wild-type allele); bottom panel shows genotyping for 504-bp neomycin gene product (only present in targeted allele). Nonspecific product of ~550 bp is commonly detected in this PCR reaction.

In Northern blot analysis, SA+/+ mice had markedly reduced amounts (~10% by densitometric analysis) of SA transcripts in the kidney and liver compared with SA+/− mice (Figures 3a and 3b). Furthermore, the transcript in the SA−/− mouse was, as expected from the absence of exons 2 and 3, smaller in size compared with that of the SA+/− mouse (Figure 3a). RT-PCR analysis with primers located in exons 1 and 4 of the SA gene confirmed this observation (Figure 3c). The amplicon from SA−/− mice RNA was ~420 bp shorter than that from SA+/− mice, equivalent to the lengths of exon 2 and 3 (422 bp). RNA from SA−/− animals had both fragments (Figure 3c). Sequencing of the fragments verified their origin from SA mRNA and confirmed that the 270-bp fragment lacks the sequence of exons 2 and 3 with exon 1 spliced directly to exon 4 (data not shown).

Western blotting studies confirmed the absence of SA protein in SA−/− mice (Figure 4). In kidney samples, cross-reactivity of the SA106 antibody to another protein called KS (see Discussion section), which is also expressed in the kidney (Figure 3a), obscured the absence of the SA protein. However, KS is not expressed in the liver (Figure 3b), and here a protein of the predicted size of ~64 kDa was consistently detected in the SA−/− mice and was completely absent in SA+/− mice (Figure 4). Prior incubation of SA antibody with SA C-terminal peptide eliminated binding to the 64-KDa band (data not shown).

Of the total number of animals genotyped during the course of the work, 155 were SA+/−, 402 SA−/−, and 176 SA+/+. These data are consistent with the expected mendelian 1:2:1 ratio (χ² = 4.05, 2 df, P = 0.132) and indicate that a lack of the SA gene does not deleteriously affect fetal survival. Likewise, direct interbreeding of SA−/− mice confirmed that these animals are fertile (data not shown). SA−/− mice appeared to be...
healthy and showed no overt phenotype. Body weight, as well as the weights of several internal organs, was similar to that of SA+/+ mice (Table). Plasma urea and creatinine levels were also similar (Table). Histological analysis of kidneys and electron microscopic analysis of proximal tubular cells showed no obvious disruption of the renal architecture of SA−/− mice and no difference compared with SA+/+ mice (data not shown). Steady-state kidney renin mRNA levels were also not different between SA+/+ and SA−/− mice (Figure 3a).

There was no difference in basal blood pressure between SA+/+ and SA−/− mice (SA+/+: 116.2±5.8 mm Hg; SA−/−: 115.6±4.3 mm Hg, P=0.565). After 4 weeks of continued normal or switch to a high salt diet, BP changes compared with baseline were as follows in the 4 groups: SA+/+ normal diet: +2.3±4.6 mm Hg; SA+/+ high salt diet: +8.8±6.3 mm Hg; SA−/− normal diet: +1.5±5.1 mm Hg; SA−/− high salt diet: +6.5±7.9 mm Hg. High salt produced a highly significant increase in BP (P=0.001), but there was no difference between the SA−/− and SA+/+ animals (P=0.340) or an interaction between genotype and salt (P=0.622).

The evolution of radiotelemetric SBP and DBP in SA+/+ and SA−/− mice under different conditions is shown in Figure 5. Again, there was no difference in either basal SBP (P=0.534) or DBP (P=0.801) between SA−/− and SA+/+ mice. Overall, there was a significant (P<0.001) and sustained effect of a high salt intake on both SBP and DBP. However, there was no difference in the response between SA−/− mice and SA+/+ mice (SA−/− ΔSBP = 7.4±1.6 mm Hg, SA+/+ ΔSBP = 8.5±2.9 mm Hg, P=0.718; SA−/− ΔDBP = 7.0±1.6 mm Hg, SA+/+ ΔDBP = 6.8±2.2 mm Hg, P=0.957, comparing the average BP in the last 2 weeks of the salt period with average basal BP). Treatment with losartan led to a significant (P<0.001) fall in BP. However, again there was no difference in response between SA−/− and SA+/+ mice (SA−/− ΔSBP = 15.5±2.1 mm Hg, SA+/+ ΔSBP = 15.7±3.5 mm Hg, P=0.965; SA−/− ΔDBP = 9.1±0.7 mm Hg, SA+/+ ΔDBP = 12.0±3.5 mm Hg, P=0.169, comparing the average BP on losartan with the average BP in the first recovery period). There was good recovery of BP in both groups of animals after cessation of losartan treatment. There was diurnal variation in BP in both groups of animals with higher BP during the night (8 PM to 8 AM, active period) than during the day. However, there was no difference between the strains for any of the periods (data not shown).

Discussion

Since its identification more than 10 years ago, several observations have suggested a potential role of SA in BP and volume homeostasis (see Introduction). However, direct investigation of such involvement has hitherto been lacking. Here, to address this issue, we report the construction and analysis of an SA−/− mouse. An alternative strategy could have
been to overexpress the gene with the use of a transgenic approach.

Our targeting strategy eliminated exons 2 and 3 of the SA gene, including the translation initiation ATG codon and the sequence for the first 65 amino acids of SA protein. Interestingly, a transcript was still produced from the disrupted SA gene, although at a markedly reduced level compared with the normal allele, suggesting either inefficient transcription of the disrupted gene or instability/inefficient processing of the aberrant transcript. Inspection of the sequence of the truncated transcript indicated that it was highly unlikely to generate a protein product. However, attempts to verify this by Western blotting initially produced a perplexing result. The antibody to the C-terminal end of SA detected a single band, of the size expected for a normal SA gene product, in the kidneys of both SA+/− and SA−/− mice. Importantly, no truncated protein was seen in SA−/− mice. This suggested that the antibody may be cross-reacting with another protein of a similar size to SA. A literature review revealed the recent identification of a protein called KS (kidney specific) of the exact size as SA and sharing 55% homology at the amino acid level. Over the epitope used to generate the SA106 antibody, 9/15 amino acids are common between the SA and KS (Figure 4), supporting the likelihood that the absence of SA in the kidneys of SA−/− mice was being obscured by the SA106 antibody binding to KS. This was confirmed by examining protein extracts from the liver, which expresses SA but does not express KS (Figure 3b and Hilgers et al14). Here, a protein of the size for SA was only detected in the livers of SA+/− mice, and again there was no evidence of a truncated protein. Taken together, these data provide definitive evidence that the SA−/− mouse does not produce SA protein.

Although SA is mainly expressed in the kidney and to a lesser extent the liver, there is a low level of expression in other tissues, notably the testes and brain. It was therefore possible that loss of SA may cause other phenotypes. However, the SA−/− mouse is overtly healthy, with no obvious disturbance of growth or behavior. With regard to the testicular expression, there was no disturbance of fertility or any selective loss of SA−/− fetuses. Consequently, the lack of any other major abnormalities made it much easier to evaluate any hemodynamic consequences of lack of SA.

Because of the increased expression of SA in the SHR, our expectation was that if SA had a role in BP regulation, then its absence was likely to result in a lower BP. However, using both indirect and direct techniques to evaluate BP, we found no effect of lack of SA on basal BP. Compensatory mechanisms could mask any effect and we therefore examined responses under different conditions. Given that SA is expressed in the proximal renal tubule, which is a site of similar size to SA. A literature review revealed the recent identification of a protein called KS (kidney specific) of the exact size as SA and sharing 55% homology at the amino acid level. Over the epitope used to generate the SA106 antibody, 9/15 amino acids are common between the SA and KS (Figure 4), supporting the likelihood that the absence of SA in the kidneys of SA−/− mice was being obscured by the SA106 antibody binding to KS. This was confirmed by examining protein extracts from the liver, which expresses SA but does not express KS (Figure 3b and Hilgers et al14). Here, a protein of the size for SA was only detected in the livers of SA+/− mice, and again there was no evidence of a truncated protein. Taken together, these data provide definitive evidence that the SA−/− mouse does not produce SA protein.

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BP effect with transfer of the SHR SA gene 20 have been observed. However, the studies in congenic strains do not provide compelling evidence that SA is not involved in the mechanism of changes in sodium intake. However, using 2 complementary high salt intake protocols, we found no difference in behavior between SA+/+ and SA−/− mice, and, specifically, there was no evidence that the BP response to salt was blunted in the SA−/− mice. Moreover, we found no evidence that activation of the renin-angiotensin system was compensating for loss of SA. Therefore, all together, the findings provide compelling evidence that SA is not involved in the regulation of either basal or salt-related BP.

A major impetus for undertaking the work described here was the observation linking the SA gene locus to hypertension in recombinant crosses involving the SHR.3–6 Moreover, the increased SA expression in the SHR is genotype-dependent,6,10 providing a possible mechanism for the linkage of the SA gene to BP.6,10 However, recent studies in which the relevant rat chromosome 1 region has been dissected in congenic strains indicate that the SA gene is unlikely to be the responsible BP QTL.20–22 Thus, both a BP effect in the absence of substitution of the SA gene21 as well as a lack of BP effect with transfer of the SHR SA gene20 have been observed. However, the studies in congenic strains do not exclude the possibility of gene-gene interactions or address whether a dramatic reduction in SA levels can affect BP. Thus, it was of interest to measure BP in gene-targeted animals totally lacking SA.

A caveat applicable to the interpretation of all gene-targeting experiments, and especially those assessing complex quantitative traits, is the possible effect of the genetic background. We assessed the loss of SA on a mouse 129 strain genetic background (see Methods), and the question of whether other backgrounds would have produced different findings remains open. Even within the 129 strain, there is evidence of genetic heterogeneity in substrains, with strains 129/Sv and 129/Ola showing variability in ∼26% of microsatellite markers.23 Since our experimental animals were derived from a partially inbred line from these 2 substrains (87.5% 129/Sv: 12.5% 129/Ola), the residual heterozygosity could be calculated to be ∼3.25% (12.5% of 26%), including that of the SA allele. This heterozygosity will have been randomly distributed in the progeny used in the experimental studies and unlikely to have influenced the findings, but, as in the case of all gene-targeting experiments, the potential impact of genetic background needs to be borne in mind in interpreting our findings.

Recent findings in vitro suggest that SA may function as a mitochondrial acyl-CoA synthetase involved in medium-chain fatty acid metabolism.24 However, the precise nature of the in vivo substrates for SA and the physiological consequences remain unclear. Interestingly, we did not find any obvious abnormalities in the number or morphology of mitochondria in the proximal tubules of SA−/− mice. Availability of a SA−/− mouse provides a potentially important resource to investigate a possible involvement of SA in energy generation.

Perspectives
Gene expression profiling is identifying an increasing number of novel genes of unknown function showing differential expression in complex diseases. Such genes need detailed analysis to confirm or exclude their role in the disease process. The SA gene represents such a gene, emerging from an early application of this approach to the field of experimental genetic hypertension. In this study, by constructing and analyzing a mouse strain lacking the SA gene product, we show that despite the indirect evidence, SA does not play a significant role in the regulation of basal or salt-related BP. Our study highlights a paradigm that will increasingly need to be pursued before a particular pathophysiological role can be definitively attributed to a novel gene.

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References
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