ORIGINAL RESEARCH

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Genetic Control of Serum Marinobufagenin in the Spontaneously Hypertensive Rat and the Relationship to Blood Pressure

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***Background*—**We have investigated serum levels of immunoreactive marinobufagenin (MBG) in 16- to 20-week-old spontaneously hypertensive rats (SHRs)-A3 and in the normotensive Wistar-Kyoto (WKY) rat strain in the absence of salt loading, and we have investigated the genetic control of serum MBG.

***Methods and Results*—**We genotyped the F2 progeny of an SHR-A3×WKY intercross using a genome-wide panel of 253 single-nucleotide polymorphism markers that were dimorphic between SHR-A3 and WKY and measured serum MBG by ELISA. Serum MBG levels were lower in SHR-A3 than WKY rats (0.39±0.07 and 1.27±0.40 nmol/L, respectively), suggesting that MBG may not play a role in the markedly divergent blood pressure measured by telemetry in rats of these 2 strains (SHR-A3 and WKY, 198.3±4.43 and 116.8±1.51 mm Hg, respectively). The strain difference in serum MBG was investigated to determine whether genomic regions influencing MBG might be identified by genetic mapping. Quantitative trait locus mapping indicated a single locus influencing serum MBG in the region of chromosome 6q12. Homozygosity of WKY alleles at this locus was associated with increased serum MBG levels. We surveyed whole genome sequences from our SHR-A3 and WKY lines, seeking coding sequence variation between SHR-A3 and WKY within the mapped locus that might explain the inherited strain difference in serum MBG.

***Conclusions*—**We identified amino acid substitution in the sterol transport protein Abcg5, present in SHR-A3, but absent in WKY, that is a potential mechanism influencing MBG levels. **(*J Am Heart Assoc*. 2017;6:e006704. DOI: 10.1161/JAHA.117.006704.)**

Key Words: cholesterol homeostasis • endocrinology • marinobufagenin • Na^+/K^+-ATPase • sitosterol

There is ongoing debate about the chemical identity of an endogenous ligand for the evolutionarily conserved cardiotonic steroid (CS; "digitalis") binding site that acts to inhibit the ion transport function of Na^+, K^+-ATPase and that may also induce cell signaling.[1] An important area where increased knowledge is needed is identification of genes involved in the regulation and biosynthesis of this proposed endocrine material. At present, there is evidence that the CSs that can be detected in mammals by several indirect assays (functional and immunologic) may have an endogenous origin from steroid biosynthesis[2–6] and may involve modification of the cholesterol side chain by CYP27A1.[7] However, studies to investigate the genetic control of circulating CS have not been reported.

Genetic approaches may offer important opportunities to refine understanding of the origin and biosynthesis of CS. They may also explain interindividual variability in measured CS levels. Knowledge of biosynthetic and regulatory genes may also help clarify uncertainty about the chemical identity of CS present in mammalian serum. This field has been shaped by an early report that the mammalian CS was chemically identical to the structurally unusual (highly hydrophilic), plant-derived CS, ouabain.[8] However, numerous independent investigations, including those using the most modern analytical chemistry methods, have shown that ouabain is not present in humans.[4,9–11] We and others have developed evidence in support of similarity between CS and the vertebrate steroid, marinobufagenin (MBG).[2,5–7,12–14] MBG is a CS aglycone synthesized by members of the Bufonidae family of true toads.[15] Studies of cultured adrenocortical cells suggest MBG is also a product of mammalian adrenocortical steroidogenesis.[2,3,5,6,13] However, definitive chemical and structural identification is incomplete. Use of cultured adrenocortical cells grown in defined media provides an opportunity to investigate endogenous origin. Also, it has provided evidence that material with MBG properties can be

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Accompanying Table S1 and Figure S1 are available at http://jaha.ahajournals.org/content/6/10/e006704/DC1/embed/inline-supplementary-material-1.pdf

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Clinical Perspective

What Is New?

- This article provides evidence that the endogenous production of the cardiotonic steroid marinobufagenin may be subject to inherited influences attributable to genetic variation in a sterol transport protein.

What Are the Clinical Implications?

- This work provides an initial genetic approach to uncover the mechanisms involved in regulation and biosynthesis of marinobufagenin.

detected and is related to cholesterol metabolism in adrenocortical cells. Specifically, we and others have shown that production of immunologically detected MBG is independent of cholesterol side chain cleavage, can be interrupted by blockade of de novo cholesterol synthesis,[2] and appears to involve modification of the cholesterol side chain by a pathway known to be involved in bile acid synthesis.[7]

In the present study, we report that immunologically detected MBG differs in abundance in the serum of inbred normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHRs). We have used genetic mapping in an SHR×WKY intercross to seek genomic loci that may be linked to such differences. The resulting mapping suggests that a narrowly defined locus on chromosome 6 influences serum MBG levels. We have examined genome sequence across SHR and WKY in this locus to identify genetic variation potentially related to serum MBG levels. We propose that the observations we have reported may be attributable to known functional genetic variation in sterol transport genes in this locus.

Methods

Animals

Studies were performed on male rats of the SHR-A3 (SHRSP/Bbb) and the normotensive WKY/Bbb inbred lines. These lines are maintained as closed colonies in our facility. The genetic integrity of the lines is verified using high-throughput genotyping of genome-wide single-nucleotide polymorphisms.[16] Animals used in these studies were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International–approved animal facility with sentinel monitoring to confirm the absence of transmissible disease in the colony. They were provided a standard rodent chow diet and drinking water ad libitum. For mapping, SHR-A3 males were crossed with WKY females to generate the F1 progeny. F1 animals were brother-sister mated to create an F2 population from which 56 males (to avoid estrus cycle fluctuations in serum steroid hormones present in females) were selected for further study. Blood was collected into serum separator tubes by direct sampling from the abdominal aorta in anesthetized (3% isoflurane in oxygen by inhalation), laparotomized animals. Serum was collected after centrifugation and stored at −80°C.

Blood Pressure

Blood pressure (BP) was obtained from 16 SHR-A3 and 15 WKY animals. BP was measured by radiotelemetry in adult animals from 16 to 18 weeks of age. BP was also measured in the F2 progeny used for serum MBG estimation by the same method. Additional F2 animals were added, subsequently increasing the number of F2 animals from which BP measurements were available to 173. Catheters were implanted under isoflurane anesthesia into the abdominal aorta above the bifurcation and below the renal arteries. Animals were allowed to recover from implantation for at least 7 days before BP measurement began. After recovery, BP was measured for 24 hours. During each 24-hour recording, period pressures were sampled for 30 seconds every 30 minutes using DataQuest ART telemetry software.

Serum MBG Measurement

An ELISA was used for serum MBG measurement. Anti-MBG antibody production and purification was described previously in detail.[17,18] Briefly, venom was collected from adult *Bufo marinus* toads, and MBG was purified by thin-layer chromatography and detected by chromatographic mobility and specific color reaction with antimony trichloride. The chemical structure of MBG was confirmed by mass spectrometry analysis. MBG-3-glycoside was synthesized, as described by Koenigs and Knorr,[19] with some modifications. The scheme of MBG-protein preparation used in our study is given in Figure S1. MBG-3-glycoside bovine serum albumin conjugate was used for rabbit immunization, and MBG-3-glycoside-RNAase conjugate was used for coating of the solid phase in ELISA. Immunization protocols, MBG antibody purification procedures, and the results of anti-MBG antibody cross-reactivity analysis, as well as the linear range and detection limits, have been previously reported.[20,21] For MBG measurements, serum samples from 10 SHR-A3, 9 WKY, and 56 F2 animals (aged 16–20 weeks) were prepared using C18 SepPak cartridges. Cartridges were activated with 10 mL of acetonitrile and washed with 10 mL water. Then, 1.0 mL of serum was applied to each cartridge, and the cartridge was rinsed with 7 mL of 25% acetonitrile, followed by extraction with 7 mL of 80% acetonitrile. The resulting extracts were dried under vacuum and stored at −80°C. Before

immunoassay, extracts were reconstituted in assay buffer and tested for their ability to inhibit the binding of rabbit anti-MBG antibody to solid-phase bound MBG (immobilized conjugate of MBG-3-glycoside to RNAse, 0.2 μg of conjugate in 0.1 mL of bicarbonate-buffered saline per well). We added 20 μL of MBG standards and unknown samples to the coated wells, followed by 80 μL of MBG antibody. After 1 hour of incubation, the wells were washed 3 times with 0.9% NaCl containing 0.05% Tween 20, after which 100 μL of secondary antibody was added (goat anti-rabbit IgG peroxidase). After 1 hour of incubation, the wells were washed 3 times and peroxidase substrate was added (TMB Microwell Peroxidase Substrate System). Optical density was read at 450 nm. The sensitivity of immunoassay was 0.0012 pmol per well. The cross-immunoreactivity of MBG antibody was as follows: MBG, 100%; digitoxin, 3.0%; bufalin, 1.0%; digoxin and cinobufagin, 1.0%; ouabain, 0.1%; and prednisone, spironolactone, proscillaridin A, progesterone, and pregnenolone, all <0.1%.

Genotyping

Single-nucleotide polymorphism genotyping was performed in DNA prepared from liver tissue in multiplex reactions using the Sequenom MassARRAY system. We selected ≈290 evenly spaced (average, ≈10Mbase between each marker) single-nucleotide polymorphism mapping markers known to be dimorphic in this cross (see Table S1 for information on marker locations throughout the genome). This resulted in successful automated single-nucleotide polymorphism genotyping calling from 253 of the selected markers. Mass spectrometry analysis of the extension reactions was performed using a Bruker Compact matrix-assisted laser desorption ionization time-of-flight mass spectrometer. The mass spectra were collected, and SpectroCALLER software was used to automatically assign the genotype calls. Genotypes were obtained for sample parental line animals (SHR-A3 and WKY) and for all F2 animals.

Genetic Mapping

Mapping with R/qtl was performed using Haley-Knott regression to test the hypothesis that a single quantitative trait locus (QTL) could be identified that influences serum MBG levels.[22,23] R/qtl was also used to estimate QTL effect sizes. The R/qtl version was 1.37-11.

Statistical Analysis

Serum MBG immunoreactive levels were obtained from 10 SHR-A3 and 9 WKY animals. Group results are described by mean±SEM. Group comparisons were performed using Student *t* tests (2 group) or using ANOVA, followed by post hoc pairwise comparisons using a Scheffé test (multigroup). Statistical analysis was performed using StatPlus:mac, Version 6.

Animal Welfare

All procedures involving the use of animals in this study were prospectively reviewed and approved by the Institutional Animal Care and Use Committee.

Results

Serum MBG levels in SHR-A3 and WKY rats were 0.39±0.07 and 1.27±0.40 nmol/L, respectively (Figure 1). Mean F2 serum MBG levels were 1.11±0.13 nmol/L. Systolic BP in SHR-A3 and WKY rats was 198.3±4.43 and 116.8±1.51 mm Hg, respectively (Figure 2). Average systolic BP in F2 animals was 144.1±1.01 mm Hg.

QTL mapping was performed to determine whether any genomic loci were linked to serum MBG levels. The resulting map is shown in Figure 3. QTL mapping provided suggestive evidence of a locus linked to serum MBG level that was centered on chromosome 6 (9.1 megabases), with a peak limit of detection score of 2.7 and the closest mapping marker being *DSgcf11040* (Table S1). We investigated the effect of inheritance of SHR-A3 alleles at this chromosome 6 QTL on estimated serum MBG levels using R/qtl. Figure 4 shows that homozygosity or heterozygosity for SHR-A3 alleles (*AA* and *AW* in Figure 4) at this chromosome 6 QTL was associated with much lower levels of serum MBG than homozygosity for WKY alleles (*WW* in Figure 4). This suggests

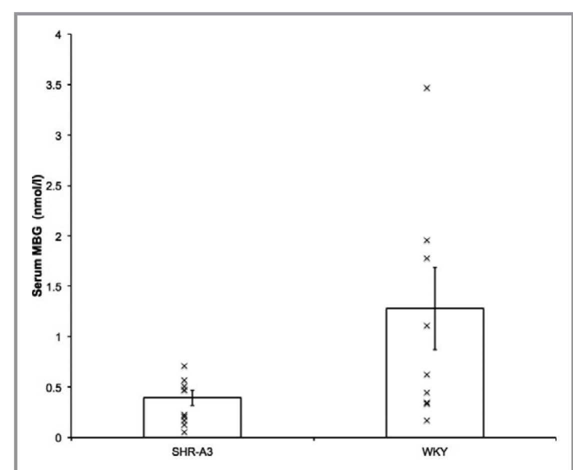

Figure 1. Marinobufagenin (MBG) levels (mean, SEM, and individual values plotted) in serum extracts from Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs)-A3 measured by ELISA (*P*<0.02 by *t* test; n=9 and n=10 for WKY and SHR-A3, respectively).

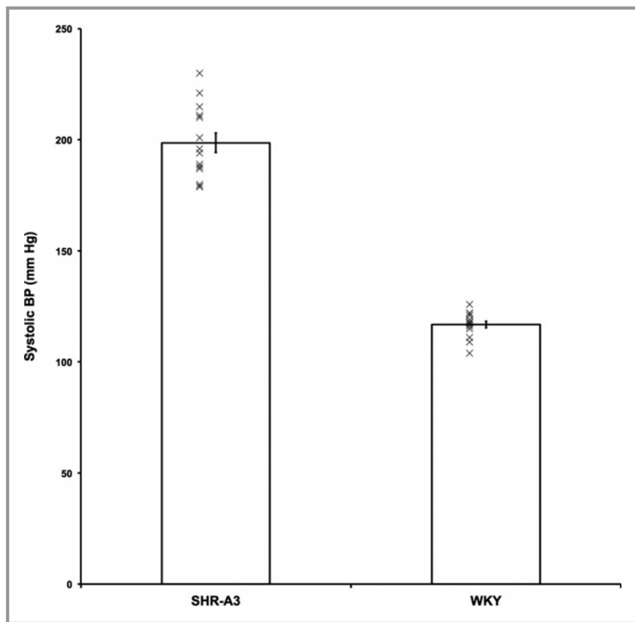

Figure 2. Systolic blood pressure (BP) levels (mean, SEM, and individual values plotted) in 16- to 18-week-old Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs)-A3 measured by telemetry ($P<0.001$ by *t* test; n=14 and n=14 for WKY and SHR-A3, respectively).

a dominant effect of inheritance of chromosome 6 genetic variation arising from SHR at this locus to decrease serum MBG.

To determine whether genetic effects on serum MBG levels might be related to BP, we examined BPs in F2 animals in relation to the inheritance of SHR-A3 (*A*) or WKY (*W*) alleles at the chromosome 6 MBG QTL. In F2 animals whose genotypes at chromosome 6 (9.0 megabases) were SHR-A3 homozygous (*AA*), SHR-A3 heterozygous (*WA*), or WKY homozygous (*WW*), systolic BP levels were as follows: *AA*, 149.3±2.80 mm Hg; *AW*, 143.3±1.25 mm Hg; and *WW*, 140.4±1.77 mm Hg (ANOVA F=4.9107; *P*=0.0085; Fcrit=3.0533; Scheffé test *P* values for *WW* versus *AA*, *P*=0.011, *WW* versus *WA*, *P*=0.521, and *WA* versus *AA*, *P*=0.066.) Thus, BP effects may also be associated with genetic variation at this QTL. However, the direction of these effects does not provide a link between increased serum MBG and BP elevation in the SHR model.

We have used next-generation whole genome sequencing to identify rat genes lying in the QTL between markers at chromosome 6 (879 568 to 24 378 026) that contain amino acid substitutions in WKY or SHR-A3, compared with the rat reference genome sequence (*Rn5*). We found only 8 genes with coding sequence variation. These genes are identified in Table. The known functions of these genes have been considered for their potential role in modulating serum MBG levels and are considered in the Discussion.

Discussion

There is extensive evidence implicating CS in the pathogenesis of various models of salt-dependent hypertension and in the potential end-organ sequelae associated with such elevation of BP.[24–29] We initiated these studies to determine whether CS might be relevant to BP levels in the widely used SHR model of hypertension. Although the SHR-A3 line does show additional elevation of BP after salt loading, severe hypertension is present in SHR in the absence of salt loading.[30] Given the association between salt-sensitive BP elevation and CS levels, it may not be surprising that the SHR model does not reveal increased endogenous CS. However, it was unexpected to find significantly lower levels of CS in SHR-A3 compared with WKY rats. This suggested an opportunity to use genetic approaches to determine whether this difference

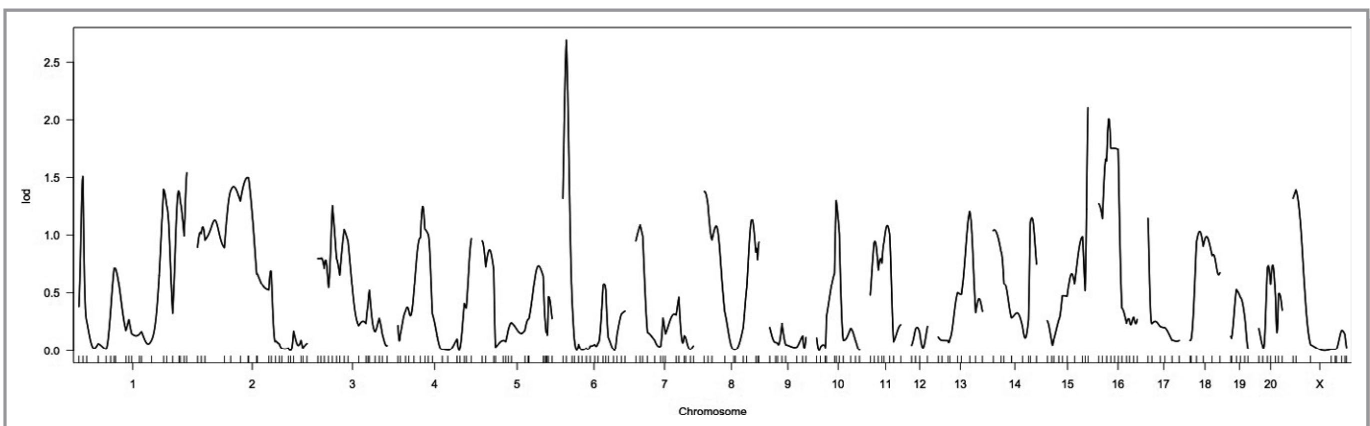

Figure 3. Limit of detection (LOD) plot of serum marinobufagenin levels in the F2 progeny of a spontaneously hypertensive rat-A3×Wistar-Kyoto intercross. The chromosome position of 253 single-nucleotide polymorphism markers is indicated on the x axis. The highest LOD score was achieved at chromosome 6 (9 megabases). Mapping was performed with R/qtl using Haley-Knott regression.

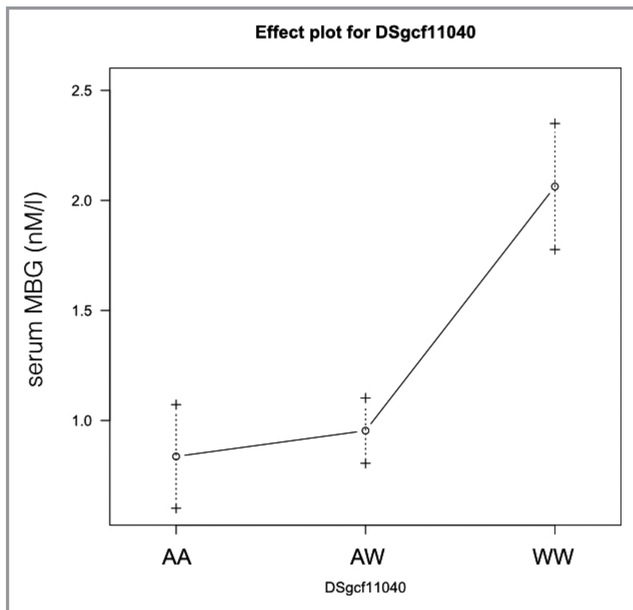

Figure 4. Effect of inheriting spontaneously hypertensive rat (SHR)-A3 (*A*) and Wistar-Kyoto (WKY; *W*) alleles at chromosome 6 (9 megabases; marker name, *DSgcf11040*). Effect sizes were estimated in R/qtl using the "effectplot" function, which estimates weighted averages.[23] Animals homozygous or heterozygous for SHR-A3 alleles (*AA* and *AW*) at *DSgcf11040* had lower levels of serum marinobufagenin (MBG) than animals homozygous for WKY alleles (ANOVA F=5.15; Fcrit=3.17; *P*=0.009; Scheffé tests: *WW* vs *AW*, *P*=0.015; *WW* vs *AA*, *P*=0.025; and *AA* vs *AW*, *P*=0.956).

Table. Genes in the MBG QTL That Contain Amino Acid Substitutions

Gene	Chromosome 6 Gene Start Position	Coding Variant	Variant Strain*
Cebpz	1 524 597	Leu418Ile	WKY
		Ser962-Cys	WKY
Prkd3	1 604 557	Asn117Ser	SHR-A3
		Ala511Thr	SHR-A3
Thumpd2	3 762 198	Ile209Val	SHR-A3
		Met542Val	SHR-A3
Haao	7 007 338	Ala267Val	SHR-A3
Thada	7 677 173	Gln427Lys	WKY
		Asn1018Ser	SHR-A3
		Ala1023Val	WKY
Abcg5	7 896 799	Gly583Cys	SHR-A3
Abcg8	7 897 005	Thr325Met	WKY
		Leu344Ser	WKY
Lrpprc	8 001 949	Asn249Ser	SHR-A3
		Ser1020Ala	WKY

MBG indicates serum marinobufagenin; QTL, quantitative trait locus; SHR, spontaneously hypertensive rat; and WKY, Wistar-Kyoto.
*Indicates which strain differs from the rat reference sequence.

in serum MBG might be determined by genetic differences between SHR and WKY rats. An opportunity resulting from this line of investigation is to identify genetic variation affecting the biosynthesis or control of CS, with the potential to yield useful insight into important aspects of CS biochemistry and endocrinology.

We have previously developed evidence concerning the nature, cellular origin, and biosynthesis of mammalian material with properties similar to CSs. We have shown that the adrenocortical cell line Y-1, grown in a defined, serum-free medium, releases into the medium a material with chromatographic and mass spectrometric properties similar to the known vertebrate CS, MBG.[2,3] MBG is an aglycone CS identified first as a defensive secretion of the parotid gland of members of the Bufonidae family, the common toads.[15] Using Y-1 cell cultures, we have demonstrated that this material arises from the pathway in which cholesterol is biosynthesized. However, it differs from known steroid hormone products of the adrenal cortex in being produced by a pathway that does not involve cholesterol side chain cleavage.[2] Recently, Fedorova and colleagues have shown that the CYP27A1-mediated modification of the cholesterol side chain, a modification that is in the pathway of bile acid formation, may be a step by which cholesterol is converted to MBG.[7]

SHR-A3 and WKY rats are inbred strains derived from outbred Wistar rats. It is well known that inbred lines derived from outbred Wistar rats may fix in homozygosity a variant of the *Abcg5* gene. This variant has important functional consequences for the handling of cholesterol and dietary phytosterols and results in increased plasma total plant sterol levels.[31] Our genome sequence analysis indicates that this *Abcg5* variant is present in inbred SHR-A3 strain, but absent in our WKY strain (Table), which is derived from WKY/Izm.[32] WKY/Izm is the only inbred WKY or SHR strain reported to lack this *Abcg5* variation.[33] Several facts point to the possibility that this *Abcg5* variation is responsible for the difference in serum MBG observed between SHR-A3 and WKY rats. First, the *Abcg5* variation maps directly under the chromosome 6 linkage peak we detected. Second, *Abcg5* is the only gene in the locus in which variation has been previously linked to steroid hormone metabolism. Third, the only other gene in the locus previously linked to sterol metabolism that contains coding variation is *Abcg8*, which is known to function as a binding partner of *Abcg5*, with each protein contributing as a half transporter to the functional sterol transporter. We found that, in the F2 progeny, the effects of the SHR-A3 allele on serum MBG levels act in the direction expected from the MBG level in the parental strains

and result in lower serum MBG levels when present in F2 animals.

Functional studies of the effects of the SHR-A3 *Abcg5* variant on sterol metabolism indicate several facts that may support a role for this variation in altered MBG levels in SHR-A3. First, the *Abcg5* mutation present in SHR-A3 has been associated with reduced adrenal cholesterol content.[34] The same phenotype of reduced adrenal cholesterol content has been observed in a more exaggerated degree with total loss of *Abcg5* and *Abcg8* function in a double-knockout mouse.[35] Furthermore, humans with homozygous functional deletion of *Abcg5* have adrenocortical insufficiency, with reduced cortisol responses to corticotropin stimulation.[36] Furthermore, phytosterolemia results in inhibition of CYP27A1,[37] an enzyme implicated in MBG biosynthesis, whereas salt loading appears to increase CYP27A1 adrenocortical abundance.[7] These findings point to a possible mechanism of reduced MBG production by the adrenal glands in SHR-A3 compared with WKY animals in this study. The abnormal sterol handling induced by the *Abcg5* mutation may reduce adrenal cholesterol availability and impede CYP27A1 activity; together, this may constrain biosynthesis of MBG in SHR-A3. Our genetic studies provide a rationale for the direction of future studies into MBG biosynthesis.

We have also found a relationship between inheritance of SHR-A3 alleles at the MBG QTL and systolic BP. The effect size on systolic BP of this locus is moderate, ≈13 mm Hg. This raises the question of whether this effect is also mediated by genetic variation in *Abcg5*. Because the standard rodent chow fed to our animals contains principally plant-derived nutrients and because the presence of the *Abcg5* mutation in SHR-A3 is sufficient to produce phytosterolemia, it is possible that altered sterol metabolism in SHR-A3 compared with WKY may contribute to disturbed BP regulation. Existing evidence suggests that phytosterolemia may affect BP levels. In F2 animals from an SHR×Sprague Dawley cross in which *Abcg5* wild-type and mutant alleles segregated, tail cuff measurements of BP indicated slightly, but not significantly, higher levels of BP in the F2 progeny inheriting homozygous mutant *Abcg5* alleles compared with those inheriting homozygous wild-type *Abcg5* alleles.[38] The magnitude of this difference in 16-week-old animals was similar to that observed between our 16- to 20-week-old SHR-A3 and WKY animals. Effects on BP of dietary phytosterol intake in WKY rats harboring the Abcg5 mutation have also been investigated. Increasing intake by changing food phytosterol content from 0.2 to 2.1 g/kg lowered cholesterol content in tissues and increased BP by ≈12 mm Hg.[39] Dietary phytosterol supplementation in stroke-prone SHR (SHR-A3) rats also resulted in increased BP and acceleration of stroke.[40] The mechanism of BP changes of phytosterols in the presence of the Abcg5 mutation has not been elucidated, but may be related to effects of phytosterols on red blood cell deformability[41] and platelet function.[42] Phytosterolemia in Abcg5/Abcg8 knockout mice is associated with platelet abnormalities, including platelet activation and microparticle formation.[43] Phytosterol loading in SHR and the resulting increase in BP are accompanied by altered renal gene expression of several genes implicated in BP.[44]

In summary, we have identified a chromosome locus that influences serum MBG levels in SHR and that contains known functional variation in the important sterol transport protein encoded by *Abcg5*. The metabolic effects of this mutation may include effects to limit the availability of substrate and enzyme activity required for MBG biosynthesis; they also may also increase BP through other mechanisms. This work lends support to the importance of cholesterol as a precursor molecule in MBG synthesis in the adrenal glands and indicates *Abcg5* as an important target for future investigation of the control of MBG production.

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Disclosures

None.

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Supplemental Material

Table S1. Markers and their positions used for SNP (single nucleotide polymorphisms) genotyping.

SNP marker ID	chromosome	position (Mbp))
gnlti56722	1	17.139
gkob1330	1	26.531
rdahlb1350	1	46.208
gnlti93476	1	61.784
gnlti01028	1	79.777
gnlti52687	1	90.431
gnlti08801	1	98.576
rdahlb1356	1	101.758
gnlti59517	1	125.888
WKYs1203	1	132.962
gnlti24582	1	139.403
DSgaf1621	1	157.604
gnlti81974	1	162.245
gkob177	1	185.342
SHRSPr1927a	1	213.369
rdahlb1630	1	220.638
WKYs1241	1	234.502
WKYs1454	1	249.482
Cpn97077	1	252.197
WKYGif1619	1	261.097
Cpn30354	1	267.130
SHRSPr1675	2	2.892
SHRSPs174	2	11.549
Cpn75992	2	20.176
SHRSPr11037	2	28.781
WKYs1422	2	48.056
gnlti91117	2	65.243
WKYOas1345	2	79.640
DahlSbr169	2	102.154
WKYr1498	2	120.104
SHRSPr1316	2	121.243
WKYOas1515	2	139.958
gnlti28177	2	141.186
WKYOas160	2	168.205
gkob1578	2	173.970
rdahlb1103	2	183.996
gkob194	2	192.803
ratcb629	2	198.234
gkob1212a	2	213.692
SHRSPr1538	2	219.012
gkob1518	2	225.693
ratcb486	2	242.876
ratca608	2	247.061
gkob1189	2	256.708
gnlti89021	3	6.445
ratcb145	3	13.643

gkob1513	3	21.453
WKYr1208	3	31.460
gkob1490	3	40.454
WKYGif1184	3	50.028
gnlti73066	3	57.503
ratca483	3	67.663
Cpn06547	3	76.607
SHRSPr1273	3	95.587
gkob1374	3	101.235
Cpn34396	3	118.129
Cpn92684	3	122.593
WKYs1575	3	126.238
gkob1320	3	139.446
WKYs1653	3	148.888
gnlti56655	3	156.297
gnlti81100	3	167.427
WKYGjf1253	4	3.713
Cpn74882	4	9.075
SHRSPr1685	4	21.334
gkob1792	4	28.965
gkob165a	4	40.690
gkob1638	4	46.591
WKYs1261a	4	55.671
WKYs1381	4	65.965
rdahlb1302	4	73.796
ratca224	4	83.360
ratca663	4	106.595
DSgc1275	4	119.414
gnlti49591	4	140.301
rdahlb1462	4	141.041
gnlti53423	4	147.616
gkob1292	4	157.496
WKYs1630	4	162.204
ratca208a	4	173.911
WKYGif1829	5	0.144
gkob1295	5	8.433
Cpn16274	5	17.516
SHRSPs1302	5	26.908
gnlti41173	5	31.072
gkob1521	5	48.841
gkob1342	5	54.801
WKYs1417	5	61.101
gkob1181a	5	68.069
SHRSPs1440	5	99.335
gkob1311	5	106.408
Cpn39002	5	108.839
gkob1337a	5	141.745

WKYs1615	5	145.032
SHRSPr1271	5	148.385
gnlti50648	5	150.352
Cpn01903	5	153.702
rdahlb1378	5	162.338
WKYr176	6	1.068
DSgcf11040	6	9.010
WKYs1663	6	22.443
Cpn73879	6	27.474
gkob1201	6	37.442
WKYr1526	6	45.740
gnlti43349	6	55.339
DSgaf1128	6	64.251
gnlti80969	6	75.043
WKYGfr1414	6	82.482
ratca431	6	94.397
WKYGjf1232	6	100.597
gnlti87595	6	106.354
DSgcf1155	6	118.530
Cpn70553	6	126.671
WKYs1104	6	136.628
SHRSPs1372	6	145.120
rdahlb1145	7	1.535
WKYs1443	7	11.598
WKYOas1463a	7	17.684
rdahlb1616	7	28.662
rdahlb1496	7	45.056
gkob1551	7	59.291
gkob1237	7	64.708
gkob1167	7	70.298
gkob1281	7	95.133
Cpn11739	7	101.412
Cpn65618	7	113.866
ratca161	7	117.117
gnlti07347	7	128.153
ratca534	7	135.139
gkob1224	8	0.966
gkob1488	8	9.921
gkob2120	8	18.990
rdahlb1212	8	48.410
SHRSPr1758	8	69.618
DSgc1111	8	73.480
gkob1164	8	80.822
gnlti54929	8	91.557
Cpn72345	8	99.572
WKYs1487	8	120.626
ratca96	8	124.882

Cpn52115	8	127.952
gnlti85693	9	24.414
SHRSPr1558	9	37.887
SHRSPr1297	9	42.237
SHRSPr1598	9	52.763
gkob124	9	61.392
SHRSPr1375	9	87.238
WKYOar1123	9	100.798
WKYs1321a	9	108.212
SHRSPr1102	10	9.202
rdahlb1251	10	18.102
WKYs1755	10	28.465
ratca65	10	31.783
SHRSPs194	10	50.587
ratca422	10	54.401
DSgaf1646	10	61.877
rdahlb1507	10	71.032
Cpn04941	10	88.105
gkob1512	10	99.462
gkob1464	10	108.586
ratca310a	11	0.258
DSgaf1726	11	9.563
DSgar155	11	18.295
rdahlb1155	11	28.129
ratca480	11	36.467
gkob1277	11	41.325
gkob172	11	54.440
rdahlb126	11	63.554
SHRSPr1554	11	80.282
SHRSPs1371	12	2.418
WKYs1582	12	11.382
ratca591	12	20.845
SHRSPr127	12	29.640
gnlti69184	12	39.299
rdahlb1110	13	8.519
WKYr1204	13	17.103
Cpn15422	13	30.915
gnlti81740	13	35.603
ratca430	13	53.175
WKYOar1256a	13	62.233
Cpn21290	13	81.321
gkob1102	13	95.095
WKYr1376	13	110.912
Cpn26240	14	2.826
WKYOas1110	14	18.900
ratca388	14	21.022
gnlti23218	14	27.803

rdahlb1187	14	44.785
ratca218a	14	45.560
Cpn55096	14	70.755
WKYr1625	14	84.593
WKYs1248	14	88.707
gnlti27885	14	103.145
SHRSPr1301	15	11.885
SHRSPs1102	15	22.224
gnlti88742	15	26.987
gkob174	15	39.063
gkob1144	15	46.730
SHRSPs1572	15	57.814
rdahlb1664	15	75.215
gkob1535	15	84.264
gkob1396	15	93.740
gkob1254	15	99.647
Cpn52335	15	106.252
WKYs1198	16	0.569
WKYr174	16	9.131
ratca254a	16	18.825
gkob1215	16	28.247
gnlti70071	16	36.796
rdahlb1205	16	45.679
WKYs1300a	16	54.121
Cpn88473	16	64.888
WKYGgr163	16	71.648
WKYr1437	16	81.655
gkob1213	16	89.728
WKYOas1201	17	2.911
Cpn54082	17	12.454
DSgaf1152	17	19.559
Cpn15864	17	28.816
WKYGjr1185	17	48.097
gnlti14648	17	58.841
gkob1218	17	75.204
gkob167	1	35.248
gkob1343	17	92.009
DSgcf1162	18	3.122
J1308870	18	6.216
WKYs1353a	18	18.061
gkob1194	18	33.755
SHRSPr1458	18	54.454
SHRSPr1494	18	72.472
gkob1612	19	5.493
gkob1120	19	7.533
WKYs1364	19	18.638
SHRSPr1649	19	27.966

gkob1192	19	37.850
gkob1133	19	45.850
ratca460	20	0.035
ratca559	20	8.901
WKYs1124	20	19.701
SHRSPs156	20	27.269
ratca621	20	38.643
WKYr1292	20	45.513
SHRSPr1498	20	54.228
SHRSPs189	X	3.967
WKYGjf11223	X	14.504
Cpn54944	X	21.255
WKYr1841	X	37.124
gnlti77424	X	55.167
rdahlb1173	X	103.023
J500540a	X	112.484
WKYGgr177	X	134.806
gkob1389	X	138.594

Figure S1. Pathway of synthesis of marinobufagenin assay reagents. **A**) Synthesis of marinobufagenin-3-glycoside. **B**) Mass spectrometry analysis of marinobufagenin-3-glucoside. **C**) Wohl degradation of glycoside ring to allow coupling to protein. **D**) Borohydride reductive coupling to bovine serum albumin.