Role of 20-HETE in the antihypertensive effect of transfer of chromosome 5 from Brown Norway to Dahl Salt-sensitive rats

Jan M. Williams¹, Fan Fan¹, Sydney Murphy¹, Carlos Schreck², Jozef Lazar³,⁴, Howard J. Jacob²,⁴, and Richard J. Roman¹

Department of ¹Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, MS and Departments of ²Physiology, ³Dermatology, and ⁴Human and Molecular Genetics Centers, Medical College of Wisconsin

Running Title: 20-HETE, hypertension, and renal disease

Corresponding Author: Richard Roman, PhD
Department of Pharmacology and Toxicology
University of Mississippi Medical Center
2500 North State Street
Jackson, MS 39272
Phone: (601) 984-1602
Fax: (601) 984-1637 E-mail: rroman@umc.edu
ABSTRACT

This study examined whether substitution of chromosome 5 containing the CYP4A genes from Brown Norway rat onto the Dahl S (SS) genetic background upregulates the renal production of 20-HETE and attenuates the development of hypertension. The expression of CYP4A protein and the production of 20-HETE was significantly higher in the renal cortex and outer medulla of SS.5BN consomic rats fed either a low salt (LS) or high salt (HS) diet than that seen in SS rats. The increase in the renal production of 20-HETE in SS.5BN rats was associated with elevated expression of CYP4A2 mRNA. MAP measured by telemetry rose from 117±1 to 183±5 mmHg in SS rats fed a HS diet for 21 days but only increased to 151±5 mmHg in SS.5BN rats. The pressure-natriuretic and diuretic responses were two-fold higher in SS.5BN rats compared to SS rats. Protein excretion rose to 354±17 mg/day in SS rats fed a HS diet for 21 days compared to 205±13 mg/day in the SS.5BN rats and the degree of glomerular injury was reduced. Baseline glomerular capillary pressure (Pgc) was similar in SS.5BN rats (43±1 mmHg) and Dahl S (44±2 mmHg) rats. However, Pgc increased to 59±3 mmHg in SS rats fed a HS diet for 7 days, while it remained unaltered in SS.5BN rats (43±2 mmHg). Chronic administration of an inhibitor of the synthesis of 20-HETE (HET0016, 10 mg/kg/day, i.v.) reversed the antihypertensive phenotype seen in the SS.5BN rats. These findings indicate that the transfer of chromosome 5 from the BN rat onto the SS genetic background increases the renal expression of CYP4A protein and the production of 20-HETE and that 20-HETE contributes to the antihypertensive and renoprotective effects seen in the SS.5BN consomic strain.

Keywords: hypertension, glomerulosclerosis, chromosome 5, 20-HETE, Dahl S rats, pressure natriuresis, renal hemodynamics, kidney
INTRODUCTION

The Dahl salt-sensitive (SS) rat is an inbred genetic model that rapidly develops severe hypertension, proteinuria, glomerulosclerosis and renal interstitial fibrosis when fed a high salt (HS) diet (4, 7-8, 23, 25, 27, 30, 38, 43). However, the genes and pathways that contribute to the development of hypertension and renal disease have yet to be identified. Previous studies from our laboratory have demonstrated that the pressure natriuretic relationship is impaired in SS rats and that this is associated with increased Cl⁻ transport in the thick ascending limb of Henle (TALH) (13, 15-16, 29, 44). They also exhibit a deficiency in the renal production of 20-HETE that contributes to the increase in loop Cl⁻ transport (13, 35, 44).

More recently, Mattson et al. demonstrated that substitution of chromosome 5 from the Brown Norway (BN) rat onto the SS genetic background (SS.5BN strain) attenuates the development of hypertension and proteinuria in a SS.5BN rats fed a high salt (HS) diet for 21 days (20) but the mechanism is unknown. Since the CYP4A genes that produce 20-HETE are located on chromosome 5 and this region has been found to cosegregate with the development of hypertension in a cross of SS and normotensive Lewis rats (34), the present study examined whether the antihypertensive and renoprotective effect of transfer of chromosome 5 from BN rats onto the SS background in the SS.5BN consomic strain is associated with upregulation of the renal expression of CYP4A protein and the formation of 20-HETE.

METHODS

General. Experiments were performed on 163 male Dahl SS/mcw (SS) and SS.5BN consomic rats that were obtained from inbred colonies maintained at the Medical College of Wisconsin and the University of Mississippi Medical Center. The SS.5BN rats were derived as
previously described using a speed congenic breeding approach (5-6) by intercrossing SS and BN rats and then backcrossing the animals with SS rats for 5-6 generations while selecting founders in each generation that remained heterozygous for BN chromosome 5. The colonies were maintained in Laboratory Animal Care Facility at both institutions, which are both approved by the American Association for the Accreditation of Laboratory Animal Care. The rats had free access to food and water throughout the study. All protocols received approval by the Animal Care Committee of both institutions.

**Protocol 1. Comparison of the expression of CYP4A protein and the metabolism of arachidonic acid in SS and SS.5BN rats.** The expression of CYP4A protein and renal metabolism of AA was compared in microsomes prepared from the renal cortex and outer medulla of 10 week old SS and SS.5BN rats that were maintained from weaning on a purified AIN-76 diet (Dyets Inc. Bethlehem PA) containing 0.4% NaCl (low salt-LS) or were switched to a AIN-76 diet containing 8.0% NaCl (high salt-HS) for 7 days prior to the experiment. We chose to examine the kidneys of these rats after only 7 days on a HS diet to avoid the effects that hypertension-induced renal injury may have on the expression of CYP4A protein and the renal metabolism of AA in the SS rats. At the end of experiments, the rats were euthanized and kidneys were collected. Microsomes were prepared from both the renal cortex and outer medulla as previously described (2). Briefly, the renal cortex (0.5 g) or outer medulla (0.3 g) were homogenized in 3 ml of a 10 mM potassium buffer (pH 7.7) containing 250 mM sucrose, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenates were centrifuged at 3,000 g for 5 min and 9,000 g for 15 min. The supernatant was centrifuged at 100,000 g for 1 h to obtain the microsomal fraction. The pellets were resuspended in the 100 mM
potassium buffer (pH 7.25) containing 20% glycerol, 1 mM dithiothreitol, and 0.1 mM PMSF, frozen in liquid N2, and stored at –80°C until assayed.

Expression of CYP4A protein. The expression of CYP4A protein was assessed in microsomes prepared from the cortex and outer medulla of SS and SS.5BN rats maintained on a LS diet from weaning or challenged with a HS diet for 7 days. The samples were separated by electrophoresis on 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose membranes and blocked overnight in a buffer containing 10% nonfat dry milk. The membranes were incubated for 2 h with a 1:4000 dilution of CYP4A primary antibody (cat. no. 299230, Daiichi Pure Chemicals, Tokyo, Japan) followed by a 1:4000 dilution of a horse radish peroxidase coupled secondary antibody (sc2020, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. The blots were developed using an enhanced chemiluminescent kit, exposed to X-ray film and the relative intensities of the bands in the 50 to 52-kDa range for CYP4A were determined using an Eagle eye imaging system (Stratagene, La Jolla, CA). Microsomes prepared from the liver of a fenofibrate treated rat were used as a CYP4A standard (1 µg loaded). The blots were reprobed for β-actin for determination of equal protein loading.

Metabolism of arachidonic acid (AA). The microsomes (0.5 mg) were incubated with a saturating concentration of AA (42 μM, Amersham Biosciences, Piscataway, NJ) and 1 mM NADPH for 30 minutes at 37°C. The incubations were stopped by acidification to pH 3.5 with formic acid and extracted twice with 3 mL of ethyl acetate after the addition of 2 ng of an internal standard, d6-20-hydroxyeicosatetraenoic acid (Cayman Chemicals, Ann Arbor, MI). The organic phase was collected and dried under N2. The samples were reconstituted with 50% methanol and water and the metabolites were separated by HPLC on a Betabasic C18 column (150x2.1 mm, 3 µm; Thermo Hypersil-Keystone, Belletonte, PA) at a flow rate of 0.2 mL/min
using an isocratic elution starting from a 51:9:40:0.01 mixture of acetonitrile:methanol:water:acetic acid for 30 minutes followed by a step change to 68:13:19:0.01 acetonitrile:methanol:water:acetic acid and water for 15 minutes. The effluent was ionized using a negative ion electrospray and the peaks eluting with a mass/charge ratio (m/z) of 319>245 (20-HETE), 325>251 (d6-20-HETE) 319>301 (HETEs and EETs), 337>319 (DiHETEs), or 325>251 (internal standard) were monitored using an Applied Biosystems 3000 triple quadrupole mass spectrometer (Foster City, CA). The ratios of ion abundances in the peaks of interest versus those seen with the internal standard were determined and compared with standard curves generated over a range from 0.5 to 10 ng for the various eicosanoids. Values are expressed as picomoles of product formed per minute per milligram of protein.

Similar experiments were performed on glomeruli and renal microvessels isolated from the kidneys of SS and SS.5BN rats fed either a LS or HS diet for 7 days by differential sieving as previously described (7, 31-32, 42). Homogenates of the isolated glomeruli were prepared and the metabolism of AA was determined as described above. Intact renal microvessels were incubated in 1 ml of a physiologic saline solution containing 1 mM NADPH for 60 minutes. The reactions were stopped with formic acid and extracted with ethyl acetate after addition of the internal standard as described above.

**Protocol 2. Real-Time PCR for assessment of the expression of CYP4A isoforms.** RNA was extracted from the renal cortex and outer medulla of SS and SS.5BN rats using Trizol (Sigma, St. Louis, MO). RNA (3 μg) was added to a 20 μl reverse transcription reaction using the AffinityScript QPCR cDNA Synthesis Kit (Stratagene, La Jolla, CA). The reactions were incubated at 42°C for 15 min and terminated by heating at 95°C for 5 min. Real-time PCR analysis for CYP4A1, 4A2, 4A3, and 4A8 isoforms was carried out as previously described using
isoform specific primers (9). β-actin was amplified in parallel as an internal control. Each sample was assayed in duplicate and C_T numbers (the cycle numbers at which reporter signals reach a threshold) were used to calculate relative mRNA expression normalized to the β-actin expression.

Protocol 3. Time course of the development of hypertension and proteinuria in SS and SS.5^BN rats. These experiments were performed on 9 week old SS and SS.5^BN rats maintained from weaning on the AIN76 diet containing 0.4% NaCl (LS). Telemetry transmitters (Model TA11PA-C40, Data Sciences International, St. Paul, MN) were implanted in the right femoral artery with the base of the transmitter placed under the skin on the right side of the rat. After a 5 day recovery period, mean arterial pressure (MAP) was recorded between 1 to 4 PM during a 3 day control period. The rats were then switched to a diet containing 8% NaCl (HS) and MAP was recorded on days 7, 14, and 21 of the HS diet. At each time point, urine was collected overnight to determine protein excretion. At the end of the study, the kidneys were weighed and collected to assess the degree of hypertrophy and fixed in a 10% buffered formalin solution. Paraffin sections (3 μm) were prepared and stained with Mason’s Trichrome stain to assess the degree of glomerular injury on approximately 30 glomeruli per section. The percentage of the glomerular capillary area filled with matrix material was scored according to the method of Raij et. al (26) on a 0-4 scale with 0 representing no injury, 2 indicating loss of 50% of glomerular capillary area and 4 representing the complete loss of capillaries.

Protocol 4. Comparison of the pressure-natriuretic response in SS and SS.5^BN rats. These experiments were performed on 9 week old SS and SS.5^BN rats. The rats were anesthetized with Ketamine (30 mg/kg, i.m., Phoenix Pharmaceutical Co., St. Joseph, MO) and Inactin (50 mg/kg, i.p., Sigma, St. Louis, MO) and catheters were placed in the femoral artery for
the measurement of MAP and in the femoral vein for an i.v. infusion of 2% BSA in a 0.9% NaCl solution at a rate of 100 µL/min. An adjustable aorta clamp was placed on the aorta above the left renal artery to regulate renal perfusion pressure (RPP). An ultrasound flow probe (Transonic System, Ithaca, NY) was placed on the left renal artery to measure renal blood flow (RBF) and a catheter was inserted into the left ureter for the collection of urine. FITC-labeled inulin (2 mg/mL, Sigma, St. Louis, MO) was added to the infusion solution for the measurement of GFR. After surgery, RPP was lowered to 100 mmHg by tightening the clamp on the aorta. After a 15 minute equilibration period, urine and plasma samples were collected during a 20-minute collection period. RPP was then increased to approximately 150 mmHg by releasing the aortic clamp and tying of the celiac and mesenteric arteries. After a 10 minute equilibration period, urine and plasma samples were recollected during a 20 min experimental period. At the end of the experiment, the left kidney was removed and weighed and the concentrations of Na⁺ and inulin in the urine and plasma samples were determined.

**Protocol 5. Measurement of the glomerular capillary pressure.** Glomerular capillary pressure (Pgc) was directly measured by micropuncture in 12 week old SS and SS.5BN rats fed either a LS or HS diet for 14 days. The measurements of Pgc were possible since the SS and SS.5BN rats used in this study have surface glomeruli. On the day of the acute experiment, the rats were anesthetized with ketamine (30 mg/kg, i.m.) and Inactin (50 mg/kg, i.p.) and catheters were placed in the femoral artery for the measurement of arterial pressure. The rats received an i.v. infusion of 1% BSA in a 0.9% NaCl solution via the jugular vein at a rate of 1.2 ml/hr. Pgc was directly measured by micropuncture using a servonull micropressure device (model 900, WPI, Sarasota, FL) as previously described (39).
Protocol 6. Measurement of the reflection coefficient of albumin ($d\sigma_{\text{Alb}}$) in isolated glomeruli. The reflection coefficient of albumin ($d\sigma_{\text{alb}}$) was measured in 1012 week-old SS and SS.5BN rats fed either a LS or HS diet for 7 and 21 days by using modifications of the Savin technique (31). The rats were anesthetized with isoflurane and a catheter was inserted into the femoral vein for injection of 75 mg/Kg of a high molecular weight (250 kDa) FITC-labeled dextran that is not filtered and remains in the glomerular capillaries. After 5 min, the kidneys were harvested and glomeruli were isolated using the sieving method in Hank’s buffer solution containing 6% bovine serum albumin (BSA) and then transferred to 80 µL fast-exchange perfusion chamber mounted on the stage of an inverted microscope (TS-100, Nikon Inc., Melville, NY). The FITC-labeled dextran in the glomeruli was imaged with the InCyt IM1 imaging system (Intracellular Imaging, Cincinnati, OH) using an excitation filter of 475 nm and an emission filter of 530 nm. $d\sigma_{\text{Alb}}$ was determined by measuring the changes in fluorescence in each glomerulus after rapidly (<2 sec) lowering the concentration of BSA in the bath from 6 to 4%. $d\sigma_{\text{alb}}$ was calculated as the measured percentage change of fluorescent intensity divided by the expected change in glomerular volume in response to a 33% decrease in oncotic pressure. A minimum of 10 glomeruli were studied from each rat and these experiments were performed using a minimum of 4 rats per strain.

Protocol 7. Effects of chronic blockade of the synthesis of 20-HETE with HET0016 on the development of hypertension and renal injury in SS.5BN rats. These experiments were performed on 9 week-old SS.5BN rats maintained from weaning on the AIN76 diet containing 0.4% NaCl. Telemetry transmitters were implanted as previously described above. A catheter also was inserted into the right femoral vein and routed subcutaneously to the scapular region and exteriorized through a Dacron-covered plastic button sutured subcutaneously over the
scapulae. The catheter was connected to a syringe pump via hydraulic swivel for i.v. infusions. After a 7 day recovery period, MAP was recorded as described above and an overnight urine sample was collected to determine baseline protein excretion. The rats were then switched to a diet containing 8% NaCl (HS) and separated into two groups: One group received an i.v. infusion of vehicle (11% sulfobutyl ether beta-cyclodextrin, CyDex, Inc., Lenexa, KS) in a 300 mM mannitol solution at a rate of 3 mL/day while the other received an i.v. infusion of N-hydroxy-N’-(4-butyl-2-methylphenyl) formamidine (HET0016) at a dose of 10 mg/kg/day, which is selective for inhibiting the formation of 20-HETE (41). MAP was recorded on days 7 and 14 of the HS diet. At each time point, urine was collected overnight to determine protein excretion.

**Statistical analysis.** Mean values ± SEM are presented. The significance of differences in control and experimental values within the same animal were determined by a paired t-test (two samples) or using an analysis of variance for repeated measures and a Holm-Sidak post-hoc test (multiple samples). The significance of differences in the mean values between groups was determined by one-way ANOVA followed by Holm-Sidak test. A $P < 0.05$ was considered to be significant.

**RESULTS**

**Protocol 1: CYP4A expression and CYP450 dependent metabolism of AA in SS and SS.5BN rats.** Comparisons of the expression of CYP4A protein in microsomes prepared from the renal cortex and outer medulla in SS and SS.5BN rats are presented in **Figures 1**. The expression of CYP4A protein was significantly higher in the renal cortex in SS.5BN rats compared to the
corresponding values observed in SS rats fed a LS or HS diet (Figures 1A). The expression of CYP4A protein fell by 40% in the renal cortex of SS rats fed a HS diet for 7 days while the expression of CYP4A protein remained unchanged in the renal cortex of SS.5BN rats. The expression of CYP4A protein was significantly greater in the outer medulla of SS.5BN rats fed a LS diet than that seen in SS rats (Figures 1B). The expression of CYP4A protein in the outer medulla was reduced in both strains when fed a HS diet for 7 days but it remained significantly higher in SS.5BN rats than in SS rats.

A comparison of the metabolism of arachidonic acid by renal cortical and outer medullary microsomes prepared from the kidneys of SS.5BN and SS rats is presented in Figure 2. The formation of 20-HETE was significantly higher in the renal cortex of SS.5BN rats compared to the corresponding values seen in SS rats fed either a LS or HS diet (Figures 2A). There was no significant difference in epoxygenase activity between the strains. The production of EETs and DiHETEs increased by 35% in the renal cortex in SS rats fed a HS diet for 7 days but it did not increase in SS.5BN rats. The production of 20-HETE in the outer medulla of the kidney was 2-fold higher in SS.5BN rats than in SS rats on either a LS or HS diet for 7 days (Figure 2B). We did not detect any epoxygenase activity in the outer medulla in either strain.

_CYP450 dependent metabolism of AA in isolated glomeruli and renal microvessels._ A comparison of the metabolism of AA by glomeruli isolated from SS and SS.5BN rats is presented in Figure 3A. The glomerular formation of 20-HETE was significantly higher in SS.5BN rats compared to SS rats fed either a LS or HS diet. No significant difference in epoxygenase activity was observed between the strains.

A comparison of the formation of 20-HETE in renal microvessels isolated from SS and SS.5BN rats fed a LS diet is presented in Figure 3B. The formation of 20-HETE was
significantly higher in renal microvessels of SS.5BN rats compared to the levels observed in SS rats fed either a LS or HS diet. Increasing salt intake also had no significant effect on 20-HETE production in the glomeruli or renal microvessels isolated from either strain.

**Protocol 2. Real-time PCR of CYP4A isoforms.** A comparison of the expression of CYP4A1, 2, 3 and 8 mRNA in the renal cortex and outer medulla of SS and SS.5BN rats is presented Figure 4. There were no differences in the expression of CYP4A1 and CYP4A3 in the cortex (Figures 4A and 4B) or outer medulla (Figures 4C and 4D) in SS and SS.5BN rats fed either a LS or HS diet for 7 days. However, the expression of CYP4A2 was elevated and the expression of CYP4A3 was reduced in both the cortex and outer medulla of SS.5BN rats as compared to SS rats fed either a LS or HS diet. The expression of CYP4A8 was lower in SS.5BN rats than SS in both the renal cortex and outer medulla whether the rats were fed either a LS or HS diet.

**Protocol 3: Time course of the development of hypertension and proteinuria in SS and SS.5BN rats.** A comparison of the time course of the development of hypertension and proteinuria in SS and SS.5BN rats fed a HS diet for 21 days are presented in Figure 5. On a LS diet (day 0), MAP was similar in both strains (≈116 mmHg) (Figure 5A). However, after 21 days on a HS diet, MAP rose to 183±5 mmHg in SS rats but only increased to 151±5 mmHg in SS.5BN rats. Protein excretion rose from 54±11 to 354±17 mg/day in SS rats fed a HS diet for 21 days as compared to 62±7 to 205±13 mg/day in the SS.5BN rats over the same period (Figure 5B).

A comparison of the degree of renal injury in SS and SS.5BN rats is presented in Figure 6. The kidneys from SS rats exhibited severe glomerular injury with mesangial expansion, glomerulosclerosis, renal interstitial fibrosis (blue color), and the formation of protein casts (red
color). The degree of renal injury was reduced in SS.5^{BN} rats and these animals only exhibited focal segmental glomerulosclerosis and mesangial matrix expansion (Figure 6A). The glomerular injury score (Figure 6B) was significantly higher in SS rats as compared to the values seen in SS.5^{BN} rats fed a HS diet for 21 days. The degree of renal hypertrophy as indicated by kidney weights was similar in SS and SS.5^{BN} rats fed a HS diet for 21 days.

Protocol 4: Comparison of the pressure-natriuretic response in SS and SS.5^{BN} rats. A comparison of the pressure-natriuretic response in SS and SS.5^{BN} rats is presented in Figure 7. Urine flow and Na⁺ excretion increased from 19±5 to 80±15 µL/min/g and 3±1 to 11±1 µEq/min/g, respectively in SS rats when RPP was increased from 100 to approximately 160 mmHg in both strains (Figures 7A and B). The pressure-natriuretic and diuretic responses were two-fold higher in SS.5^{BN} rats as compared to SS rats. RBF was similar in SS and SS.5^{BN} rats at 100 mmHg (Figures 7C). However, RBF rose 20% in SS.5^{BN} rats when RPP was increased and was significantly higher than that seen in SS rats. Baseline GFR was not significantly different in SS and SS.5^{BN} rats at 100 mmHg (Figure 7D). GFR increased significantly in both strains when RPP was increased to approximately 160 mmHg and was significantly higher in SS.5^{BN} rats as compared to SS rats.

Protocol 5: Measurement of glomerular capillary pressure (Pgc). The relationship between MAP and Pgc in SS and SS.5^{BN} rats fed either a LS or HS diet for 7 days is presented in Figure 8. MAP measured under Inactin anesthesia during the micropuncture experiments were similar in SS and SS.5^{BN} rats maintained on a LS diet (Figure 8A). However, switching rats to a HS diet for 7 days increased MAP in SS rats, while it remained unaltered in SS.5^{BN} rats. Directly measured Pgc was similar in both strains fed a LS diet (Figure 8B). Pgc rose by 15
mmHg in SS rats fed a HS diet whereas it did not increase in SS.5\textsuperscript{BN} rats. Thus, Pgc was significantly higher in SS rats fed a HS diet than in SS.5\textsuperscript{BN} rats.

\textit{Protocol 6: Glomerular permeability to albumin.} A comparison of $\delta$alb in glomeruli of SS and SS.5\textsuperscript{BN} rats fed a LS or HS diet for 7 and 21 days is presented in Figure 9. Baseline $\delta$alb was similar in SS and SS.5\textsuperscript{BN} rats fed a LS diet. SS rats exhibited a marked decrease in $\delta$alb values when fed a HS diet for 7 and 14 days while we did not observe any changes in $\delta$alb in SS.5\textsuperscript{BN} rats.

\textit{Protocol 7: Effects of chronic blockade of the synthesis of 20-HETE on the development of hypertension and renal injury in SS.5\textsuperscript{BN} rats.} The effects of chronic treatment with an inhibitor of the synthesis of 20-HETE, HET0016, on the development of hypertension and proteinuria in SS.5\textsuperscript{BN} rats are presented in Figure 10. MAP rose from 117±2 to 129±2 mmHg in control SS.5\textsuperscript{BN} rats fed a HS diet for 14 days (Figure 10A). In contrast, MAP rose from 117±3 to 155±3 mmHg in SS.5\textsuperscript{BN} rats treated with HET0016 which is similar to the increase in MAP seen in SS rats fed a HS diet for 14 days (Figure 5A). Moreover, SS.5\textsuperscript{BN} rats treated with HET0016 displayed progressive proteinuria that was significantly greater than that seen in vehicle treated rats (591±92 vs. 207±32 mg/day, respectively) (Figure 10B).

\textbf{DISCUSSION}

Previous studies have indicated that the renal formation of 20-HETE is reduced in the outer medulla of SS rats and that this contributes to enhanced Cl\textsuperscript{−} transport in the TALH, resetting of the pressure natriuretic relationship and the development of hypertension. However, it remains to be determined whether the changes in the renal formation of 20-HETE are due to unique sequence variants in the CYP4A isoforms in SS rats or secondary changes in neural and
hormonal factors that influence the expression of these genes. Recently, Mattson et al. reported that substitution of chromosome 5 from the BN rat onto the SS genetic background markedly inhibited the development of hypertension and albuminuria via an unknown mechanism (20). Since all of the genes of the CYP4A family responsible for the formation of 20-HETE are located on chromosome 5, the present study examined whether the antihypertensive effect in the SS.5\textsuperscript{BN} strain is associated with upregulation of the renal expression of CYP4A protein and formation of 20-HETE. Substitution of chromosome 5 from the BN rat increased the expression of CYP4A protein and production of 20-HETE in the kidneys of SS.5\textsuperscript{BN} rats as compared to SS rats fed either a LS or HS diet. Real-time PCR studies indicate that this is associated with an increase in the renal expression of the CYP4A2 mRNA. The pressure-natriuretic response was markedly improved and the increases in arterial pressure and proteinuria in response to a HS diet were significantly reduced in SS.5\textsuperscript{BN} rats than the values observed in SS rats. Moreover, the progression of hypertension-induced renal injury in the SS rats fed a HS diet was associated with elevations in Pgc and glomerular permeability to albumin, both of which were significantly lower in SS.5\textsuperscript{BN} rats. Finally, chronic blockade of the formation of 20-HETE with HET0016 reversed the antihypertensive and renoprotective effects seen in SS.5\textsuperscript{BN} rats and MAP rose to a similar extent as that seen in SS rats fed a high salt diet for 14 days (Figure 5A). Moreover, in previous studies we reported that blockade of the synthesis of 20-HETE has no additional effect on MAP in SS rats (28, 41).

The observation that substitution of chromosome 5 from BN into the SS genetic background increases the renal formation of 20-HETE and reduces arterial pressure in response to a HS diet supports the hypothesis that a deficiency in the renal formation of 20-HETE contributes to the development of hypertension in SS rats. Interestingly, we observed a decrease
in the expression of CYP4A protein and an increase in the formation of 20-HETE in the outer medulla of SS.5BN rats when fed a HS diet for 7 days. It is possible that a greater proportion of the CYP4A protein is inactive in the SS.5BN strain on a LS diet in the outer medulla. Indeed, there is evidence that nitric oxide (24) and carbon monoxide (1, 3) that is present in the outer medulla binds to the heme of CYP4A proteins and inhibits the formation of 20-HETE. Nonetheless, our finding that increasing the renal formation of 20-HETE in this case by transfer of chromosome 5 reduces salt sensitivity of blood pressure in SS.5BN rats is consistent with previous data indicating that induction of the synthesis of 20-HETE in the kidney with fibrates opposes the development of hypertension in SS rats and other models of hypertension (30, 33, 43). In contrast, the increase in 20-HETE formation in renal microvessels in the SS.5BN strain would be expected to increase vascular tone and promote the development of hypertension. However, we believe that the upregulation of 20-HETE in the renal outer medulla to inhibit Cl⁻ transport in the TALH predominates and overcomes any influence that increase of 20-HETE in the renal microvessels might have to reduce renal blood flow and GFR and promote the development of hypertension in the SS.5BN strain. Indeed, one might speculate that the increase in 20-HETE production in the vasculature might only serve to enhance renal myogenic and tubuloglomerular feedback responsiveness to prevent the development of glomerular injury and be renoprotective rather than to be prohypertensive. A recent study from our laboratory demonstrated that Pgc increases in SS rats fed a HS diet and that this is associated with the development of proteinuria and glomerular disease (41). In the current study, we examined whether the transfer of chromosome 5 from the BN rat into the SS genetic background would prevent the rise in Pgc when fed a HS diet. We found that after 7 days on a HS diet when both SS and SS.5BN rats exhibited a similar 15 mmHg increase in arterial pressure, Pgc increased
markedly in SS rats, but it remained unaltered in the SS.5\textsuperscript{BN} strain. This was associated with an increase in glomerular permeability to albumin and development of more severe proteinuria and renal injury in SS rats than was seen in SS.5\textsuperscript{BN} rats. Chronic blockade of the formation of 20-HETE with HET0016 reversed the antihypertensive and renoprotective effects seen in the SS.5\textsuperscript{BN} consomic strain. In contrast, we have previously demonstrated the chronic inhibition of 20-HETE has no effect on either arterial pressure (28, 41) or the degree of proteinuria and renal injury in SS rats (41). The mechanism for an increase in Pgc in SS rats fed a HS diet remains to be determined. However, from the current study, we observed the formation of 20-HETE in the renal microvessels was reduced in SS rats compared to that seen in SS.5\textsuperscript{BN} rats when fed either a LS or HS diet. Thus a deficiency in the formation of 20-HETE in the renal microvessels may impair both tubuloglomerular feedback (TGF) and/or myogenic responsiveness of the afferent arteriole in SS rats to elevations in arterial pressure making the kidney more susceptible to hypertension induced renal injury. 20-HETE is expressed in afferent arterioles and has been reported to play an important role in the myogenic response by regulating vascular reactivity secondary to inhibition of Ca\textsuperscript{2+}-activated K\textsuperscript{+} (K\textsubscript{Ca}) channels. It also modulates tubuloglomerular feedback responsiveness probably by affecting vascular reactivity to adenosine through modulation of K\textsubscript{Ca} channel activity (45). Indeed, this hypothesis is consistent with previous observations that the myogenic response (37) and dynamic autoregulation of RBF is impaired in Dahl S rats (14) and that TGF responsiveness is decreased when these rats are fed a HS diet (40).

Besides a potential effect on renal hemodynamics, we recently observed that acute blockade of the formation of 20-HETE markedly increased glomerular permeability to albumin and that this response can be reversed by exogenous administration of a 20-HETE mimetic (7,
In the present study, the glomerular formation of 20-HETE was 2-fold higher in SS.5^{BN} rats compared to values observed in SS rats fed either a LS or HS diet. Moreover, we observed that the albumin reflection coefficient (\(\partial\sigma_{Alb}\)), an indicator glomerular permeability, decreased in SS rats fed a HS diet while it remained unchanged in SS.5^{BN} rats. These data are consistent with the view that the relative deficiency in the glomerular formation of 20-HETE contributes to the loss of barrier function of the glomerulus of SS rats and the development of proteinuria.

Previous studies have indicated that the CYP4A region of chromosome 5 cosegregates with the development of hypertension in a F2 cross of SS and Lewis rats (34). The present findings that transfer of chromosome 5 from BN rats to the SS genomic background increases the expression of CYP4A protein and the formation of 20-HETE suggests there may be a sequence variant in one of the CYP4A isoforms that contributes to the strain difference in the formation of 20-HETE. The present finding that the expression of the CYP4A2 was elevated in SS.5^{BN} rats fed either a LS or HS diet than in SS rats suggests that this may be the isoform of interest.

In summary, the results from the current study suggest that substitution of chromosome 5 from the BN rat onto the SS genetic background attenuated the development of hypertension and the rise in Pge and glomerular permeability to albumin in SS.5^{BN} consomic rats fed a HS diet. The antihypertensive and renoprotective effects seen in the SS.5^{BN} rat was associated with increases in the renal formation of 20-HETE and the expression of CYP4A protein and CYP4A2 mRNA. Moreover, chronic blockade of the formation of 20-HETE rescued the hypertensive phenotype in the SS.5^{BN} rat. Overall, these findings further support the view that a deficiency in the renal formation of 20-HETE contributes to the development of salt dependent hypertension and they are important given the results of recent studies indicating that mutations in CYP4A11
and CYP4A22 are associated with the development of hypertension and cardiovascular disease in human population studies (10-12, 17-19, 21-22, 36). These results also suggest that studies in the SS rat along with the SS.5^BN consomic can serve as a useful model to better determine how alterations in formation of 20-HETE contributes to the development of hypertension, proteinuria and renal injury.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants HL29587, HL36279, and a United Negro College Fund/Merck Postdoctoral Science Research Fellowship awarded to JMW.
FIGURE LEGENDS

**Figure 1.** Comparison of the relative expression of CYP4A protein in the renal cortex (Panel A) and outer medulla (Panel B) in Dahl salt-sensitive (SS) and SS.5BN rats fed either a low salt (LS) or high salt (HS) diet for 7 days. Numbers in the bars indicate the number of rats studied per group. Values are means ± SE. * indicates a significant difference from the LS value within the same strain. † indicates a significant difference from the corresponding value in SS rats.

**Figure 2.** Comparison of the metabolism of AA in the renal cortex (Panel A) and outer medulla (Panel B) in Dahl salt-sensitive (SS) and SS.5BN rats fed either a low salt diet (LS) or a high salt diet (HS) for 7 days. Numbers in the bars indicate the number of rats studied per group. Values are means ± SE. * indicates a significant difference from the corresponding value in rats fed a LS diet within the same strain. † indicates a significant difference from the corresponding value in SS rats.

**Figure 3.** Comparisons of the metabolism of AA in glomeruli isolated from Dahl salt-sensitive (SS) and SS.5BN rats fed either a low salt (LS) or a high salt (HS) diet for 7 days (Panel A) and the formation of 20-HETE in renal microvessels isolated from Dahl salt-sensitive (SS) and SS.5BN rats fed either a low salt (LS) or a high salt (HS) diet for 7 days (Panel B). Numbers in the bars indicate the number of rats studied per group. Values are means ± SE. * indicates a significant difference from the corresponding value in rats fed a LS diet within the same strain. † indicates a significant difference from the corresponding values in the SS rats.

**Figure 4.** Comparison of the expression of CYP4A mRNA in the renal cortex (Panels A and B) and outer medulla (Panels C and D) in Dahl salt-sensitive (SS) and SS.5BN rats fed either a low salt (LS) diet or a high salt (HS) diet for 7 days. Numbers in parentheses indicate the number of
rats studied per group. Values are means ± SE. † indicates a significant difference from the corresponding value in SS rats.

**Figure 5.** Time course of the development of hypertension (Panel A) and proteinuria (Panel B) in Dahl salt-sensitive (SS) and SS.5BN rats fed a high salt (HS) diet for 21 days. Numbers in parentheses indicate the number of rats studied per group. Values are means ± SE. † indicates a significant difference from the corresponding value in the SS rats.

**Figure 6.** Comparison of the degree of glomerular injury in Dahl salt-sensitive (SS) and SS.5BN rats fed a high salt (HS) diet for 21 days. Numbers in the bars indicate the number of rats studied per group. Values are means ± SE. † indicates a significant difference from the corresponding value in the SS rats.

**Figure 7.** Comparison of the changes in urine flow rate (Panel A), Na⁺ excretion (Panel B), renal blood flow (RBF) (Panel C), and glomerular filtration rate (GFR) (Panel D) in response to elevations in renal perfusion pressure (RPP) in Dahl salt-sensitive (SS) and SS.5BN rats. Values are means ± SE. * indicates a significant difference from corresponding values in the kidneys perfused at a low RPP (100 mmHg) within the same strain. † indicates a significant difference from corresponding values measured in the SS rats.

**Figure 8.** Comparison of the measurement of mean arterial pressure (MAP) (Panel A) and glomerular capillary pressure (Pgc) (Panel B) in Dahl salt-sensitive (SS) and SS.5BN rats fed a low salt diet (LS) or a high salt diet (HS) for 14 days. Numbers in the bars indicate the number of glomeruli and rats studied per group. Values are means ± SE. * indicates a significant difference from the corresponding value in rats fed a LS diet within a strain. † indicates a significant difference from the corresponding value in the SS rats.
Figure 9. Comparison of the measurement of the dilutional glomerular albumin reflection coefficient (dσAlb) in Dahl salt-sensitive (SS) and SS.5BN rats fed a low salt diet (LS) or a high salt diet (HS) for 7 and 21 days. Numbers in the bars indicate the number of glomeruli and rats studied per group. Values are means ± SE. † indicates a significant difference from the corresponding value in the SS rats.

Figure 10. Effects of the chronic administration of an inhibitor of the formation of 20-HETE, HET0016 (10 mg/kg/day, i.v.), on mean arterial pressure (MAP) (Panel A) and protein excretion (Panel B) measured in SS.5BN rats fed a high salt diet (HS) for 14 days. Numbers in the bars indicate the number of rats studied per group. Values are means ± SE. † indicates a significant difference from the corresponding value in SS.5BN rats treated with vehicle.
REFERENCES


Figure 1. 20-HETE, hypertension, and renal disease

A

Renal cortex

B

Renal outer medulla

[Images of Western blots and bar graphs showing fold differences in expression of CYP4A and β-actin in different conditions: SS-LS, SS.5BN-LS, SS-HS, SS.5BN-HS, STD.]

Fold difference

<table>
<thead>
<tr>
<th>Condition</th>
<th>SS-LS</th>
<th>SS.5BN-LS</th>
<th>SS-HS</th>
<th>SS.5BN-HS</th>
<th>STD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal cortex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP4A</td>
<td>1.0</td>
<td>1.5</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>1.0</td>
<td>1.5</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

Fold difference

<table>
<thead>
<tr>
<th>Condition</th>
<th>SS-LS</th>
<th>SS.5BN-LS</th>
<th>SS-HS</th>
<th>SS.5BN-HS</th>
<th>STD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal outer medulla</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP4A</td>
<td>1.0</td>
<td>1.5</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>1.0</td>
<td>1.5</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.

20-HETE, hypertension, and renal disease

A

Renal cortex

CYP450 activity (pmoles/min/mg)

SS

SS.5BN

20-HETE

EETs & DiHETEs

B

Renal outer medulla

20-HETE formation (pmoles/min/mg)

SS

SS.5BN

LS HS

LS HS

6 6 6 6

6 6 6 6

6 4 4 4
Figure 3.

20-HETE, hypertension, and renal disease

**A**

**Glomeruli**

CYP450 activity (pmoles/min/mg)

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>SS.5&lt;sup&gt;BN&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>HS</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

EETs & DiHETEs

**B**

**Renal microvessels**

20-HETE formation (pmoles/min/mg)

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>SS.5&lt;sup&gt;BN&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>HS</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>
Figure 4. 20-HETE, hypertension, and renal disease

A. Low Salt Diet
   Renal cortex

B. High Salt Diet
   Renal Cortex

C. Low Salt Diet
   Renal outer medulla

D. High Salt Diet
   Renal outer medulla
Figure 5.

20-HETE, hypertension, and renal disease

A

MAP (mmHg)

SS (26)  SS.5BN (13)

B

Protein excretion (mg/day)

SS (26)  SS.5BN (13)

Days on High Salt Diet
Figure 6. 20-HETE, hypertension, and renal disease

A

SS

SS.5

B

Glomerular Injury Score

(120,4)

(120,4)

SS

SS.5

BN

BN
Figure 7. 20-HETE, hypertension, and renal disease

A

Urine flow rate (μL/min/g)

- SS (5)
- SS5MN (5)

B

Na⁺ excretion (μEq/min/g)

- SS (5)
- SS5MN (5)

C

RBF (mL/min/g)

- SS (5)
- SS5MN (5)

D

GFR (μL/min/g)

- SS (5)
- SS5MN (5)
Figure 8. 20-HETE, hypertension, and renal disease

A

MAP (mmHg)

* LS

† HS

(21,5) (33,6) (22,4) (22,4)

SS SS.5^BN

B

Pgc (mmHg)

* LS

† HS

(21,5) (33,6) (22,4) (22,4)

SS SS.5^BN
Figure 9. 20-HETE, hypertension, and renal disease
Figure 10. 20-HETE, hypertension, and renal disease

A

MAP (mmHg)

Vehicle (6)
HET0016 (5)

†

B

Protein excretion (mg/day)

Vehicle (5)
HET0016 (4)

†

0 100 200 300 400 500 600 700

0 2 4 6 8 10 12 14 16

Days on High Salt Diet