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Introgression of Brown Norway *CYP4A* Genes onto the Dahl Salt-Sensitive Background Restores Vascular Function in SS-5^{BN} Consomic Rats

Kathleen M. Lukaszewicz
Marquette University, kathleen.lukaszewicz@marquette.edu

John R. Falck
University of Texas Southwestern Medical Center at Dallas

Vijaya L. Manthati
University of Texas

Julian H. Lombard
Medical College of Wisconsin

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Introgression of Brown Norway CYP4A genes on to the Dahl Salt-sensitive Background Restores Vascular Function in SS-5^{BN} Consomic Rats

Kathleen M. Lukaszewicz
Department of Physiology, Medical College of Wisconsin, Milwaukee, WI
John R. Falck
Department of Biochemistry, University of Texas Southwestern Medical School, Dallas, TX
Vijaya L. Manthati
Department of Biochemistry, University of Texas Southwestern Medical School, Dallas, TX
Julian H. Lombard
Department of Physiology, Medical College of Wisconsin, Milwaukee, WI

Abstract

The present study tested the hypothesis that the Dahl SS (salt-sensitive) rat has vascular dysfunction due, in part, to the up-regulation of the CYP4A/20-HETE (cytochrome P450 ω-hydroxylase 4A)/20-hydroxyeicosatetraenoic acid) system. To assess the role of vascular 20-HETE, SS rats were compared with SS-5^{BN} consomic rats, carrying *CYP4A* alleles on chromosome 5 from the normotensive BN (Brown Norway) introgressed on to the SS genetic background. Cerebral arteries from SS-5^{BN} rats had less CYP4A protein than arteries from SS rats fed either NS (normal-salt, 0.4% NaCl) or HS (high-salt, 4.0% NaCl) diet. ACh (acetylcholine)-induced dilation of MCAs (middle cerebral arteries) from SS and SS-5^{BN} rats was present in SS-5^{BN} rats fed on either an NS or HS diet, but absent in SS rats. In SS rats fed on either diet, ACh-induced dilation was restored by acute treatment with the CYP4A inhibitor DDMS (*N*-methylsulfonyl-12,12-dibromododec-11-enamide) or the 20-HETE antagonist 20-HEDE [20-hydroxyeicosa-6(*Z*),15(*Z*)-dienoic acid]. The restored response to ACh in DDMS-treated SS rats was inhibited by L-NAME (N^Gnitro-L-arginine methyl ester) and unaffected by indomethacin or MS-PPOH [*N*-methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide]. Vascular relaxation responses to the NO donor $C_5FeN_6Na_2O$ were intact in both SS and SS-5^{BN} rats and unaffected by the acute addition of DDMS, indicating that the vascular dysfunction of the SS rat is due to a reduced bioavailability of NO instead of failure of the VSMCs (vascular smooth muscle cells) to respond to the vasodilator. Superoxide levels in cerebral arteries of SS-5^{BN} rats [evaluated semi-quantitatively by DHE (dihydroethidium) fluorescence] were lower than those in the arteries of SS rats. These findings indicate that SS rats have an up-regulation of the CYP4A/20-HETE pathway resulting in elevated ROS (reactive oxygen species) and reduced NO bioavailability causing vascular dysfunction.

CLINICAL PERSPECTIVES

- Recent studies have reported an association between increased urinary 20-HETE excretion, endothelial dysfunction and elevated blood pressure in humans.
- The present study used Dahl SS (salt-sensitive) rats, a genetic rodent model of salt-sensitive hypertension in humans, and a unique consomic rat strain carrying CYP4A alleles from the salt-insensitive Brown Norway rat to investigate the role of 20-HETE in contributing to vascular dysfunction in salt-sensitive hypertension. The experiments revealed a direct relationship between 20-HETE and vascular dysfunction in the Dahl SS rat.
- Owing to the intimate relationship between vascular function and cardiovascular health, the present findings could provide important clues to increase our understanding of the role of the CYP4A/20-HETE system in contributing to the changes in vascular function occurring in human hypertension and/or salt-sensitive subjects.

INTRODUCTION

Endothelial dysfunction is the failure of the endothelial cell layer to maintain vascular homoeostasis, resulting in increasing vascular constriction and reduced vascular relaxation to vasodilator stimuli. Increased levels of ROS (reactive oxygen species) in the endothelium not only compromise NO (nitric oxide)-dependent vasodilatation, but also overwhelm the antioxidant, anti-inflammatory and anti-proliferative properties of NO in the vessel wall. Endothelial dysfunction is associated with hypertension [1,2] and many other CVDs (cardiovascular diseases) [3] and has been shown to be an early prognosticator

of future adverse cardiovascular-related incidents (including myocardial infarction, stroke and death) [3,4].

20-HETE (20-hydroxyeicosatetraenoic acid) is a vasoconstrictor metabolite of arachidonic acid formed in VSMCs (vascular smooth muscle cells) through the action of CYP4A (cytochrome P450 ω-hydroxylase 4A). The CYP4A/20-HETE system has been implicated in the development of hypertension in humans and animals [5–8]. Studies on human subjects have shown an association between genetic variants in CYP4AF, a human gene that forms 20-HETE, and an increase in both urinary excretion of 20-HETE and MAP (mean arterial pressure) [5,6]. In animal models, SHR (spontaneously hypertensive rats) [7] and androgen-induced hypertensive rats [9] have elevated vascular 20-HETE and high BP (blood pressure) that can be ameliorated with a CYP4A inhibitor. Cyp4a14 knockout mice further demonstrate the role of the CYP4A/20-HETE pathway in the development of hypertension as these mice develop severe hypertension as a result of increased expression of the Cyp4a12 isoform in the kidney and enhanced 20-HETE production [8].

In addition to the pro-hypertensive effects of 20-HETE, the CYP4A/20-HETE pathway has also been associated with endothelial dysfunction. Ward et al. [10] demonstrated an association between elevated urinary 20-HETE excretion and endothelial dysfunction in humans. Similar to reports in hypertensive rat models [7,9], S–D (Sprague–Dawley) rats receiving a CYP4A2-carrying adenovirus demonstrate increased BP, reduced ACh (acetylcholine)-induced vascular relaxation and attenuated NO production [11]. In Dahl SS (salt-sensitive) rats, the constriction of skeletal muscle arterioles in response to elevated PO_2 (partial pressure of O_2) is potentiated by increased dietary salt, an effect that can be abolished with 20-HETE inhibition [12].

The present study tested the hypothesis that an up-regulation of the CYP4A/20-HETE system plays a direct role in vascular dysfunction of the SS rat through the production of ROS and the subsequent decrease in NO bioavailability. Earlier studies showed that a 3-day HS (high-salt) diet causes an up-regulation of CYP4A mRNA in SS cremasteric arterioles [12], and in mesenteric arteries from S–D rats [13], suggesting an association between increased dietary salt and elevated 20-HETE production in resistance arteries. Although it is clear that salt can activate the CYP4A/20-HETE pathway, SS rats also exhibit vascular dysfunction when they are normotensive and maintained on an NS (normal salt) diet. As such, increases in the CYP4A/20-HETE system may be independent of dietary sodium [14–16]. In order to determine the direct effects of salt on the CYP4A/20-HETE pathway independent of changes in arterial pressure, the present study evaluated the effect of short-term (3 days) elevated dietary salt intake, as the Dahl SS rat does not demonstrate salt-induced pressure changes within this limited time frame [16]. In addition, we evaluated vascular function in a novel consomic rat strain (SS-5BN) carrying CYP4A alleles from the BN (Brown Norway) rat in the Dahl SS genetic background that exhibits dramatic attenuation of salt-sensitivity of BP [17].

The SS-5BN rat is a strain developed by the PGA (Program for Genomic Applications) group at the MCW (Medical College of Wisconsin) as part of a consomic panel of rats in which single chromosomes from the BN rat were introgressed individually on to the genetic background of the Dahl SS rat using marker-assisted selection [18]. As reported by Cowley et al. [18], consomic rat strains allow investigation into the role of specific genes and chromosomes in controlling physiological traits with reduced genetic variability. For this reason, the SS-5BN consomic rat, carrying CYP4A genes on chromosome 5 from the

BN rat, is an excellent control animal for the investigation of the role of CYP4A and 20-HETE in vascular dysfunction in the Dahl SS rat. Specifically, the SS-5BN consomic rat has ~95% genetic homology with the Dahl SS rat, but exhibits protection from salt-induced BP elevations [17]. We hypothesized that these rats would also have protection from the vascular dysfunction present in the SS rat due to a decreased contribution of the CYP4A/20-HETE pathway.

MATERIALS AND METHODS

Experimental groups

Male SS (SS/JrHsd/Mcwi) and SS-Chr 5^{BN}/Mcwi (SS-5^{BN}) rats 8–10 weeks old were fed on either NS (0.4% NaCl; Dyets) from weaning or switched to an HS diet (4.0% NaCl; Dyets) for 3 days prior to the experiments, with water *ad libitum*. The MCW Institutional Animal Care and Use Committee approved all the protocols.

Isolated vessel experiments

The animals were anaesthetized with intramuscular injection containing (in mg/kg of body weight): ketamine (75.0), acepromazine (2.5) and anased (10.0). MCAs (middle cerebral arteries) were cannulated as described previously [19] and internal diameter was measured using television microscopy. Vessels lacking active tone at rest were excluded from the study.

Responses to ACh (10^{-10}–10^{-5} mol/l) and $C_5FeN_6Na_2O$ (10^{-12}–10^{-4} mol/l) were determined before and after the treatment with DDMS (*N*-methylsulfonyl-12,12-dibromododec-11-enamide, 50 μmol/l) or 20-HEDE [20-hydroxyeicosa-6(*Z*),15(*Z*)-dienoic acid, 1 μmol/l]. In another experiment, responses to ACh before and after incubation with L-NAME (N^G-nitro-L-arginine methyl ester; 100 μmol/l) to inhibit NOS, indomethacin (1 μmol/l) to inhibit cyclo-oxygenase, or MS-PPOH [*N*-methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide; 100 mmol/l] to inhibit epoxygenases were recorded in DDMS-treated MCA from SS rats and control MCA from SS-5^{BN} rats. Additional vessels were incubated without inhibitors as a time control.

At the end of the experiment, the maximum diameter was determined by adding H_2O_2 (1.76 mmol/l) to the superfusate to achieve maximum dilation. Active resting tone (%) was calculated as $[(D_{max}-D_{rest})/D_{max}]\times100$, where D_{max} is the maximum diameter in the presence of H_2O_2 and D_{rest} is the resting control diameter.

Western blotting

CYP4A protein expression in cerebral arteries was assessed by Western blotting as described previously [13,20,21] using the CYP4A1/A2/A3 antibody (sc-53247; Santa Cruz Biotechnology). Expression of eNOS (endothelial nitric oxide synthase) (610296; BD Biosciences), peNOS (phospho-eNOS) (612393;BD Biosciences), Cu/Zn-SOD (copper/zinc superoxide dismutase) (Enzo Life Sciences), Mn-SOD (manganese SOD) (Assay Designs) and EC-SOD (extracellular SOD) (sc-3222; Santa Cruz Biotechnology) were also assessed using Western blotting. Relative intensity of the bands was quantified and normalized to a loading control (β-actin) using a computer-based densitometer system and Image-Quant software (Molecular Dynamics).

DHE (dihydroethidium) fluorescence

Vascular ROS levels in basilar arteries were assessed semi-quantitatively using DHE [22]. Basilar arteries were used as a substitute for MCA, as both vessels demonstrate an NO-dependent dilation to ACh [23], and the larger diameter allows for improved cross-sectioning. In these experiments, the arteries were isolated and incubated for 1 h in PSS heated to 37° with DHE (5 μmol/l) for the final 15 min. The vessels were cut into 10 μm transverse sections and imaged with a Nikon Eclipse TS100 microscope equipped with a ×20 objective, a 540-nm excitation filter, a 605-nm emission filter (Chroma Technology) and QImaging Regiga-2000R digital camera. Fluorescence of multiple images of each artery was quantified using ImageJ software and background fluorescence was subtracted from the fluorescence value of the basilar artery ring [22].

Statistical analysis

Data are summarized as means±S.E.M. For comparisons of two groups, an unpaired Student's *t*test was used. For all concentration–response curves, differences between multiple groups at each concentration were determined using a one-way ANOVA. Differences between individual means following ANOVA were evaluated using a post-hoc Student–Newman–Keuls test. A probability level of $P<0.05$ was considered to be statistically significant.

RESULTS

Arterial BP and vessel characteristics

The PGA at the MCW generated SS-5^{BN} rats and performed phenotyping protocols, including assessment of conscious BP in the parental and consomic strains. SS-5^{BN} consomic rats are protected from salt-induced increases in MAP (130±2.6 mmHg) following a 3-week HS (8.0% NaCl) diet compared with the SS rat (177±2.5 mmHg). The present study utilized a 3-day HS diet of 4.0% NaCl because SS rats remain normotensive within this time frame [16,24] and thus BP differences between the two rat strains should not affect our results. Active resting tone (Table 1) was unaffected by the HS diet or any of the inhibitors in either strain; the only exception being HS-fed MCA of SS rats treated with 20-HEDE; which may be due to an increased contribution of 20-HETE in SS rats fed on an HS diet. Nonetheless, the consistency of active resting tone between treatment groups indicates changes in the magnitude of vascular relaxation in different experimental groups are independent of differences in resting tone and do not reflect a pre-existing constriction.

Table 1 MCA diameters and resting tone

Values are means±S.E.M. *$P<0.05$, significantly different from NS- and HS-fed SS rats in all categories, except NS-fed SS rats without inhibitor; †$P<0.05$, significantly different from NS- and HS-fed SS rats in the presence of either MS-PPOH or 20-HEDE; ‡$P<0.05$, significantly different from every other treatment group in the same category. INDO, indomethacin.

Experimental group	Number (*n*)	Maximum diameter (μm)	Resting diameter (μm)	Active tone (%)
SS NS	8	256±2.9†	129±11.3	50±4.0
SS HS	8	248±5.2	152±5.0	40±2.4
SS NS+DDMS	8	240±1.8	145±8.0	39±3.2
SS HS+DDMS	8	238±2.4	135±5.6	43±2.1

SS NS+20-HEDE	6	236±4.2	145±9.3	39±1.9
SS HS+20-HEDE	6	232±1.9	175±6.7‡	25±1.3‡
SS NS+DDMS+L-NAME	8	240±1.8	140±6.7	42±2.7
SS HS+DDMS+L-NAME	8	238±2.4	133±6.8	44±2.6
SS NS+DDMS+INDO	6	240±1.9	143±3.5	40±1.1
SS HS+DDMS+INDO	6	237±1.7	139±5.1	41±2.0
SS NS+DDMS+MS-PPOH	6	233±2.4	130±1.9	44±1.0
SS HS+DDMS+MS-PPOH	6	233±2.0	135±3.8	42±1.7
SS-5^{BN} NS	8	265±5.4*	140±6.6	47±2.5
SS-5^{BN} HS	8	260±7.2*	137±2.6	47±1.3
SS-5^{BN} NS+DDMS	8	265±5.4*	133±4.4	49±2.4
SS-5^{BN} HS+DDMS	8	260±7.2*	139±6.6	47±1.3
SS-5^{BN} NS+L-NAME	6	252±4.0	131±5.9	47±1.5
SS-5^{BN} HS+L-NAME	6	249±4.7	132±5.7	47±2.7
SS-5^{BN} NS+INDO	5	249±7.6	134±6.9	46±2.4
SS-5^{BN} HS+INDO	5	246±5.7	141±7.7	43±3.0

Effect of CYP4A inhibition and 20-HETE antagonism on vascular relaxation

MCAs from NS-fed (Figure 1A) or HS-fed (Figure 1B) SS rats failed to dilate in response to the endothelium-dependent vasodilator ACh. Vascular relaxation to ACh was restored by inhibiting 20-HETE production with DDMS in SS rats fed on either an NS or HS diet, and also by treatment with the 20-HETE antagonist 20-HEDE.

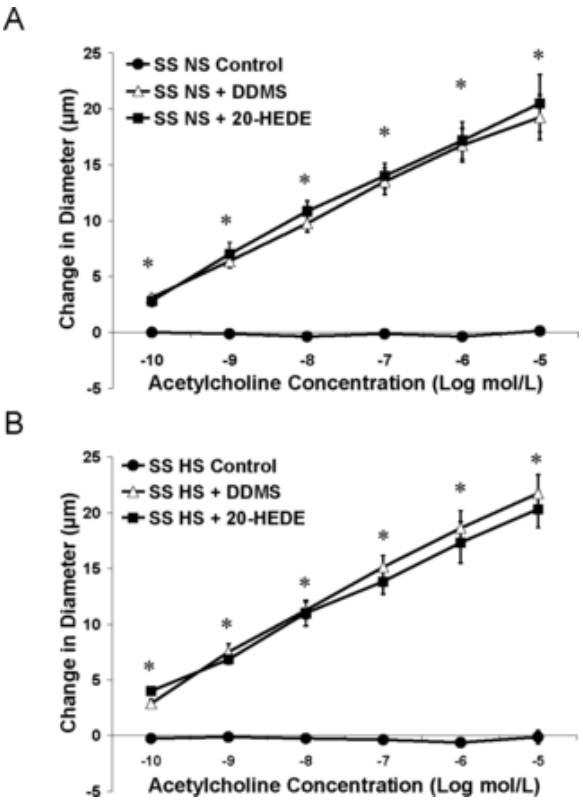

Figure 1 Response to ACh in MCA of SS rats fed on an NS (A) or HS (B) diet with or without the acute addition of DDMS or 20-HEDE

Data are expressed as the mean±S.E.M. change in diameter from baseline (µm) *P<0.05, significant difference from response in the absence of inhibitor, same diet.

Mechanisms of restored vascular relaxation

The responses of DDMS-treated MCA from SS rats (with or without the NOS inhibitor L-NAME, the cyclo-oxygenase inhibitor indomethacin, or the epoxygenase inhibitor MS-PPOH) are shown in Figure 2. The restored dilation in response to ACh in DDMS-treated MCAs from SS rats fed on an NS (Figure 2A) or HS diet (Figure 2B) was eliminated by L-NAME, demonstrating an NO-dependent response. Vessel responses to ACh did not utilize either the cyclo-oxygenase or epoxygenase pathways, as their respective inhibitors did not affect the magnitude of vasodilation.

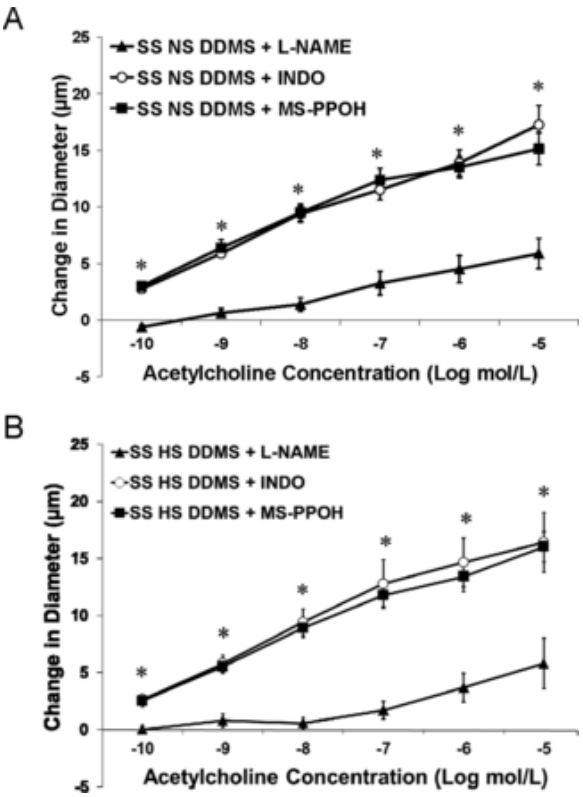

Figure 2 Response to ACh in DDMS-treated MCA of SS rats fed on an NS (A) or HS (B) diet with or without the L-NAME, indomethacin or MS-PPOH (*n*=8 for all groups)

Data are expressed as the mean ±S.E.M. change in diameter from baseline (µm) **P*<0.05, significant difference from L-NAME-treated arteries.

Effect of BN *CYP4A* alleles on vascular relaxation

Introgressing *CYP4A* genes from the BN rat into the SS genetic background restored vascular responses to ACh in SS-5^{BN} rats fed on either an NS or HS diet; and this dilation was unaffected by DDMS (Figure 3A). The ACh-induced response in these vessels was NO-dependent, as the response was blocked by L-NAME and unaffected by indomethacin (Figure 3B). Time control experiments (results not shown) demonstrated no effect of the incubation period on vascular responses to ACh in any group. MCAs from both SS and SS-5^{BN} rats fed on an NS and HS diet showed robust dilation to the NO donor $C_5FeN_6Na_2O$, with no additional effect of DDMS (Figure 4), showing vascular dysfunction in the SS rat is not due to a failure of VSMCs to respond to NO.

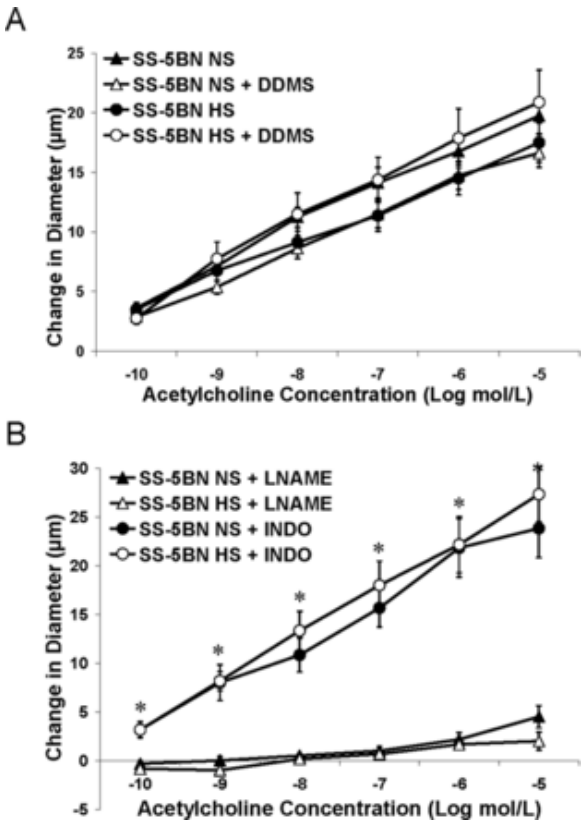

Figure 3 Response to ACh in MCA of SS-5^{BN} rats fed on an NS or HS diet with or without acute addition of DDMS (A) or either L-NAME or indomethacin (B)

Data are expressed as the mean ±S.E.M. change in diameter from baseline (µm) *P<0.05, significant difference from L-NAME-treated vessels.

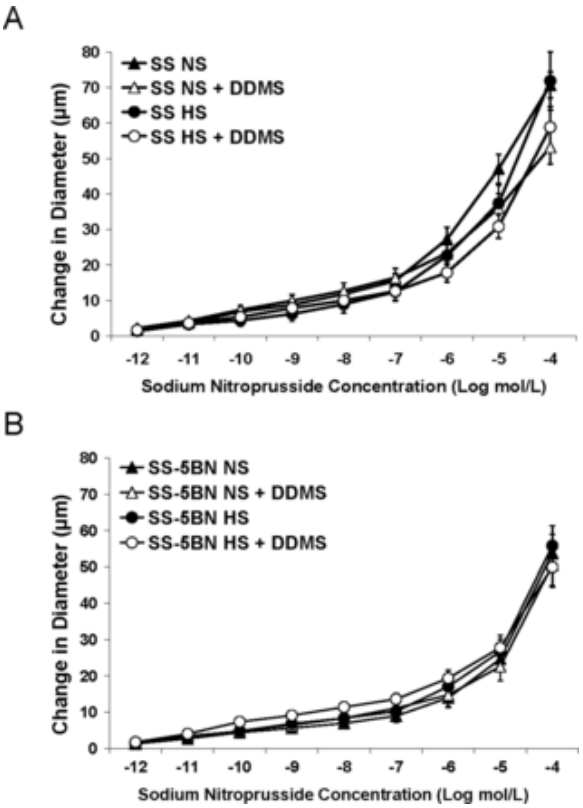

Figure 4 Response to $C_5FeN_6Na_2O$ (10^{-12}–10^{-5} M) in isolated MCAs of Dahl SS rats (A) and SS-5^{BN} consomic rats (B) fed on an NS or HS diet (*n*=12 in all groups) with or without the acute addition of DDMS (50 µM) to the perfusate and superfusate

Data are expressed as the mean±S.E.M. change in diameter from baseline (µm)

CYP4A enzyme expression in cerebral arteries from SS and SS-5^{BN} rats

CYP4A protein expression was significantly lower in cerebral arteries from SS-5^{BN} rats compared with arteries from SS rats fed on either NS (Figure 5A) or HS (Figure 5B). CYP4A expression was unaffected by changes in dietary salt intake in either animal strain (Figures 5C and 5D).

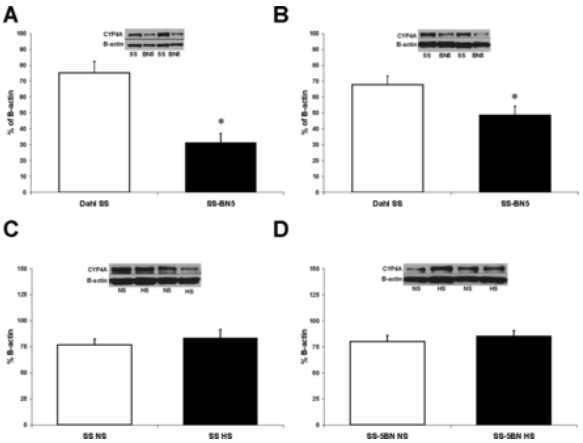

Figure 5 Western blots comparing CYP4A protein (50 kDa) expression in cerebral arteries from (A) NS-fed SS rats compared with SS-5^{BN} rats, (B) HS-fed SS and SS-5^{BN} rats (C) NS compared with HS in SS rats, and (D) NS compared with HS in SS-5^{BN} rats (*n*=8 for all groups)

Data are presented as a percentage of β-actin expression. *P<0.05, significant difference from cerebral vessels from SS, same diet.

ROS levels, SOD expression and eNOS expression

Consistent with the hypothesis that the vascular effects of 20-HETE are mediated via oxidative stress, ROS levels evaluated semi-quantitatively via DHE fluorescence were significantly higher in basilar arteries of SS rats compared with those of SS-5^{BN} rats fed on the same diet. ROS levels in HS-fed SS rats were also significantly higher than those in SS rats fed on an NS diet (Figure 6). Western blotting revealed no difference in antioxidant protein expression (Mn-SOD, Cu/Zn-SOD and EC-SOD; Figures 7A, 7B and 7C respectively) or in peNOS/eNOS expression (Figure 7D) in any of our treatment groups; suggesting vascular dysfunction in SS rats compared with SS-5^{BN} rats is not due to alterations in antioxidant defences or in the activity of eNOS.

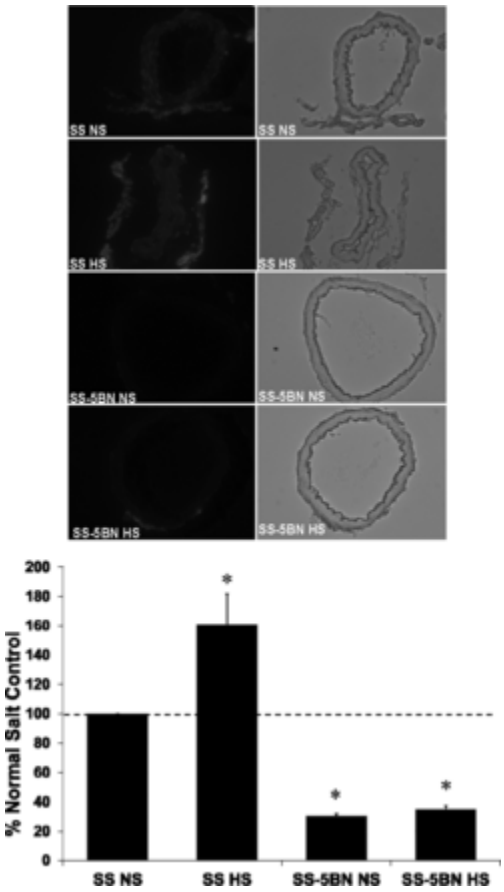

Figure 6 Semi-quantitative evaluation of ROS levels via DHE fluorescence in basilar arteries from NS- and HS-fed SS and SS-5^{BN} rats (n=6 for all groups)

Upper panel, representative fluorescent and bright field images of basilar artery cross-sections (10 µm thickness) from SS and SS-5^{BN} rats. Lower panel, vascular ROS levels expressed as fluorescence units in DHE-treated basilar artery cross-sections. Data are expressed as the mean±S.E.M. percentage of raw fluorescence units in NS-fed SS arteries. *P<0.05, significant difference from control NS-fed SS rats.

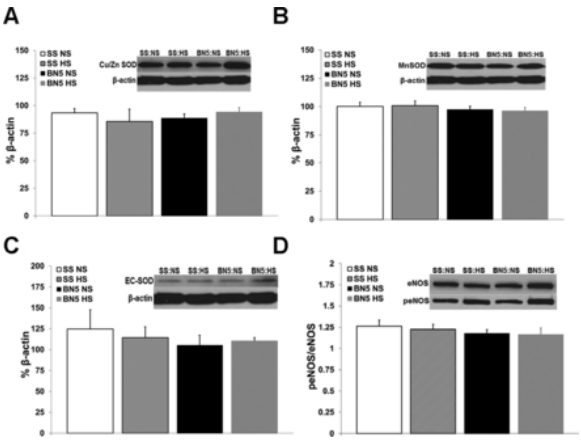

Figure 7 Western blot analysis of protein expression of (A) Cu, Zn-SOD, (B) MnSOD, (C) EC-SOD and (D) eNOS and peNOS in cerebral vessels from Dahl SS and SS-5^{BN} consomic rats fed on either an NS or HS diet

Data are presented as a percentage of β-actin (**A**–**C**) or as a ratio of the percentage of β-actin of p-eNOS to the percentage of β-actin of eNOS (*n*=4 for all groups).

DISCUSSION

The present study shows that multiple interventions that interrupt the CYP4A/20-HETE pathway ameliorate the severe vascular dysfunction that exists in the cerebral arteries of Dahl SS rats fed on either a normal or an HS diet. Specifically, pharmacological interventions that either inhibit the catalytic function of CYP4A enzymes or antagonize the actions of 20-HETE both restored vascular relaxation in cerebral resistance arteries of Dahl SS rats. In a similar fashion, genetic substitution of chromosome 5 carrying the *CYP4A* alleles from the BN rat on to the genetic background of the SS rat (the SS-5^{BN} consomic rat) reduced the influence of this pathway by decreasing vascular CYP4A protein expression, and this was accompanied by a restoration of endothelium-dependent vascular relaxation in response to ACh. One limitation of the present study is the inability of the current methodology, which assesses 20-HETE production under optimum conditions of PO_2 and substrate availability, to evaluate differences in 20-HETE production under normal physiological conditions. As such, it is also conceivable that differences in CYP4A enzyme activity (in addition to differences in the expression of CYP4A enzymes in the arteries) may also exist in the two strains. Nonetheless, the findings of the current study suggest that up-regulation of the CYP4A/20-HETE pathway plays a direct role in the endothelial dysfunction that exists in the SS rat fed on an NS or HS diet. In this regard, the beneficial effects of chromosomal substitution and pharmacological interruption of the CYP4A/20-HETE pathway on vascular function in rats fed on an NS diet are especially important because they indicate that the CYP4A/20-HETE pathway may play an important role in contributing to vascular dysfunction in SS rats, even prior to the onset of hypertension.

Previous studies have shown that increased levels of ROS are important contributors to vascular dysfunction in SS rats [22,25]. For example, cerebral vascular relaxation in response to ACh and reduced PO_2 in SS rats fed on either an NS or HS diet can be restored by scavenging the superoxide with tempol [22,25]. In the present study, we utilized DHE fluorescence to evaluate vascular ROS levels. Although this approach has its limitations compared with other methods that provide a better quantitative evaluation of superoxide levels, for example HPLC determination of 2-hydroxyethidium, which is more feasible in other tissues [26–28], it still provides a valuable, albeit semi-quantitative,

estimation of vascular ROS levels. In these experiments, we found that genetic manipulation of CYP4A protein expression via chromosome substitution in the SS-5^{BN} consomic rat led to a decrease in vascular oxidant stress concomitant with a restoration of endothelium-dependent vasodilation to ACh in animals fed on either a normal or an HS diet. The latter findings suggest that impaired endothelial function in the SS rats and the beneficial effects of inhibiting 20-HETE production on vascular reactivity are secondary to a reduction in ROS. The latter interpretation is consistent with published findings showing that 20-HETE can increase vascular ROS via several mechanisms including: (i) the normal catalytic process of 20-HETE formation by CYP4A enzymes [29–31]; (ii) 20-HETE-induced NADPH oxidase activation [9,32–34]; and (iii), uncoupling of eNOS [35,36]. Since eNOS expression and phosphorylation of eNOS were similar in SS and SS-5^{BN} rats and were unaffected by the HS diet, the most probably explanation for our findings is that reduced 20-HETE levels and/or CYP4A enzyme activity restore NO bioavailability by preventing the destruction of NO by superoxide anions, rather than via any effect on eNOS expression or activation.

Current sequence analysis (http//www.rgd.mcw.edu) has identified a non-synonymous SNP (single nucleotide polymorphism) predicted to be damaging in the *CYP4A*8 allele of the SS rat that may affect expression of the other CYP4A isoforms. A similar phenomenon has been observed in the CYP4A14-knockout mouse [8], in which the knockout of the *CYP4A14* gene results in increased ω-hydroxylase activity [37], increased urinary excretion of 20-HETE and elevated MAP due to secondary up-regulation of the *CYP4A12* gene [8,37]. This raises the possibility that one or more of the other CYP4A isoforms are up-regulated in the Dahl SS rat due to a non-functional *CYP4A8*allele. This could provide a possible explanation for elevated CYP4A protein expression in cerebral arteries from Dahl SS rats fed on either a normal or an HS diet compared with arteries from SS-5^{BN}consomic rats, which do not carry the mutation.

Multiple studies have shown that the Dahl SS rat is susceptible to heightened oxidative stress due to reduced antioxidant defence mechanisms, specifically in the myocardium [38], kidney [39,40] and vasculature [22]. In the present study, there were no differences in expression of any of the SOD enzymes between SS and SS-5^{BN} rats fed on either diet. Owing to the pro-oxidant capabilities of 20-HETE, it is probable that the up-regulation of the CYP4A/20-HETE pathway operating in conjunction with reduced antioxidant defences in the SS genetic background leads to increased vascular oxidant stress in SS rats compared with SS-5^{BN} consomic rats. We believe that the most probable reason for the restoration of endothelium-dependent vascular relaxation in SS-5^{BN}consomic rat is the reduced expression of CYP4A enzymes, resulting in lower levels of ROS that do not overpower antioxidant defence mechanisms that are compromised in the SS genetic background due, at least in part, to the chronically low AngII (angiotensin II) levels resulting from impaired regulation of the renin allele (found on chromosome 13) [22,25].

It is important to discuss the contrasting roles of 20-HETE in the pathogenesis of hypertension in the vasculature compared with the kidney, with specific reference to these two rat strains. In renal physiology, 20-HETE is generally considered to be anti-hypertensive due to its tubular effects promoting natriuresis [41,42]. Deficiencies in renal 20-HETE production have been associated with sodium retention and SS hypertension in both human [43] and animal studies [44–46]. In fact, Dahl SS rats have inadequate 20-HETE production in the outer medulla [47], which can be ameliorated by introgressing BN chromosome 5 into the SS genetic background (the SS-5^{BN} consomic rat) [48]. Those observations contrast with the increased expression of CYP4A enzyme protein in the vasculature that we observed in the present study. This is probably due to local differences in the regulation of *CYP4A* gene expression in the kidney and vasculature. For example, it has been shown that human hypertensive subjects have

significantly higher amounts of plasma 20-HETE but a significantly lower urinary excretion of 20-HETE, an indirect indicator of renal 20-HETE production. Local control over 20-HETE synthesis may be a result of differences in the expression of certain receptors responsible for CYP4A enzyme activation within the kidney compared with the vasculature (e.g. receptors for either AngII or endothelin-1) [49] or due to local differences in signal transduction pathways. Regardless of these potential underlying mechanisms, our laboratory and others have consistently demonstrated that elevated 20-HETE production in the vasculature is deleterious and contributes to the development and progression of hypertension [11,13,50].

One very striking observation in the present study is that the HS diet failed to eliminate endothelium dependent dilation to ACh in arteries of the SS-5^{BN} consomic rats. This finding is in contrast with the ability of the HS diet to eliminate endothelium-dependent dilation in arteries of salt-insensitive S–D rats [51], mice [52] and SS-13^{BN} consomic rats carrying a normally functioning renin allele from the BN rat in the SS genetic background [24]. This raises the crucially important question of the relative role of the CYP4A/20-HETE pathway in contributing to salt-induced vascular impairment compared with cerebral vascular dysfunction independent of salt intake (e.g. in SS rats). Studies in mesenteric arteries of S–D rats have shown that an HS diet up-regulates *CYP4A* mRNA and protein expression, and that inhibiting CYP4A restores vascular relaxation response to reduced PO_2 [13]. By contrast, the present study shows no difference in CYP4A protein expression in the MCA of SS rats fed on either an NS or HS diet; and pharmacological blockade of the CYP4A/20-HETE pathway with either DDMS or 20-HEDE restores vascular function in both NS- and HS-fed SS rats. The latter findings indicate that CYP4A enzymes contribute to overall vascular dysfunction in the SS rat, independent of dietary salt intake. This observation may have important significance in the light of previous studies [14,15,24,25] demonstrating that vascular relaxation mechanisms are lost in the MCA of SS, even when the animals are normotensive and maintained on an NS diet.

One possible explanation for a role of the CYP4A/20-HETE pathway in vascular dysfunction independent of dietary salt in SS rats is a contribution of ROS to the regulation of CYP4A enzyme expression. A plausible mechanism by which ROS could exert a regulatory influence over the CYP4A/20-HETE pathway is indirect, via the effect of superoxide on NO. The superoxide anion reacts with NO at a rate three times higher than the interaction between superoxide and SOD, which has a major effect on the basal levels of NO [53]. NO inhibits CYP4A activity by forming a ferrous–nitrosyl complex at the haem-binding site of the CYP4A enzyme [54], and it has also been shown to decrease CYP4A protein expression [55,56]. In the present study, both CYP4A protein expression and ROS levels are increased in arteries from both NS- and HS-fed SS rats compared with the SS-5^{BN} consomic rats. Previous studies indicate that elevated ROS levels in the arteries of Dahl SS rats reduce the bioavailability of NO within the vasculature [14,25]. As a result, the inhibitory influence of NO on the CYP4A/20-HETE pathway would be absent in arteries of Dahl SS rats, which could explain the up-regulation of the CYP4A/20-HETE pathway independent of dietary salt intake in Dahl SS rats.

Consomic rat models provide an experimental animal with nearly identical genetic homology with the target animal (SS) while substituting genetic material from a normotensive rat strain (BN). However, there are still limitations to the conclusions that can be drawn from the data collected from these animals, as chromosome 5 carries BN alleles other than the *CYP4A* genes, which may contribute to the observed differences in the SS compared with the SS-5^{BN} consomic rat in the present study. Nonetheless, the SS-5^{BN} consomic rat is still an excellent animal model to test the present hypothesis that the CYP4A enzyme and 20-HETE contribute to vascular dysfunction in the SS rat, as they

demonstrate a significant reduction in the salt-sensitivity of BP [17] and lack the vascular dysfunction observed in their close genetic counterpart, the SS rat. The improved vascular function observed in SS-5^{BN} mirrors the restored vasodilator responses in MCA from SS in the presence of the CYP4A inhibitor DDMS and the 20-HETE antagonist 20-HEDE. As noted above, cerebral arteries from SS-5^{BN} also have a significantly lower expression of CYP4A protein, supporting the hypothesis that the restoration of endothelium-dependent vascular relaxation in cerebral arteries of the SS-5^{BN} rats reflects a reduced contribution of the CYP4A/20-HETE system to vascular regulation.

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AUTHOR CONTRIBUTION

Kathleen Lukaszewicz performed the experiments, analysed the data, wrote the primary draft of the paper, edited and revised the paper, and approved the final version of the paper. John Falck provided conceptual guidance for the CYP4A/20-HETE inhibitors and antagonists and CYP450 expoxygenase inhibitor, synthesized and provided the CYP4A inhibitors and antagonists and the CYP450 epoxygenase inhibitors, and approved the final version of the paper. Vijaya Manthati synthesized and provided the CYP4A inhibitors and antagonists and the CYP450 epoxygenase inhibitors, and approved the final version of the paper. Julian Lombard directed the experiments, evaluated the data, edited and revised the paper, and approved the final version of the paper.

Abbreviations: ACh, acetylcholine; AngII, angiotensin II; BN, Brown Norway; BP, blood pressure; Cu,Zn-SOD, copper/zinc superoxide dismutase; CYP4, cytochrome P450; DDMS, N-methylsulfonyl-12,12-dibromododec-11-enamide; DHE, dihydroethidium) fluorescence; EC-SOD, extracellular SOD; eNOS, endothelial nitric oxide synthase; 20-HEDE, 20-hydroxyeicosa-6(Z),15(Z)-dienoic acid; 20-HETE, 20-hydroxyeicosatetraenoic acid; HS, high-salt; L-NAME, NG-nitro-L-arginine methyl ester; MAP, mean arterial pressure; MCA, middle cerebral artery; MCW, Medical College of Wisconsin; Mn-SOD, manganese SOD; MS-PPOH, N-methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide; NS, normal salt; peNOS, phospho-eNOS; PGA, Program for Genomic Applications; PO_2, partial pressure of O_2; ROS, reactive oxygen species; S–D, Sprague–Dawley; SS, salt-sensitive; VSMC, vascular smooth muscle cell

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