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# ROLE OF VASCULAR REACTIVE OXYGEN SPECIES IN REGULATING CYTOCHROME P450-4A ENZYME EXPRESSION IN DAHL SALT-SENSITIVE RATS

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## Abstract

**Objective—**The potential contribution of CYP4A enzymes to endothelial dysfunction in Dahl salt-sensitive (SS) rats was determined by comparison to SS-5$^{BN}$ consomic rats having chromosome 5 carrying CYP4A alleles from the Brown Norway (BN) rat introgressed into the SS genetic background.

**Methods—**The following experiments were performed in cerebral arteries from HS-fed SS and SS-5$^{BN}$ rats ± the SOD inhibitor DETC and/or the superoxide scavenger Tempol: 1) endothelial function was determined via video microscopy ± acute addition of the CYP4A inhibitor DDMS or Tempol; 2) vascular oxidative stress was assessed with DHE fluorescence ± acute addition of DDMS, L-NAME, or PEG-SOD; and 3) CYP4A protein levels were compared by Western blotting.

**Results—**In DETC-treated SS-5$^{BN}$ and HS-fed SS rats: 1) DDMS or Tempol ameliorated vascular dysfunction 2) DDMS reduced vascular oxidative stress to control levels; 3) Chronic Tempol treatment reduced vascular CYP4A protein expression; and 3) combined treatment with Tempol and L-NAME prevented the reduction in CYP4A protein expression in MCA of HS-fed SS rats.

**Conclusion—**The CYP4A pathway plays a role in vascular dysfunction in SS rats and there appears to be a direct role of reduced NO availability due to salt-induced oxidant stress in upregulating CYP4A enzyme expression.

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**Author Contributions:**

KML: Performed experiments, analyzed data, wrote primary draft of manuscript, edited and revised the manuscript, approved final version of manuscript.

JRF: Provided conceptual guidance for CYP4A/20-HETE inhibitors and antagonists; synthesized and provided cytochrome CYP4A inhibitors and antagonists, approved final version of manuscript.

MPP: Synthesized cytochrome CYP4A inhibitors and antagonists; approved final version of manuscript.

JHL: Directed experiments, evaluated data, edited and revised the manuscript, approved final version of manuscript.

**Keywords**

Endothelial Dysfunction; 20-HETE; Oxidant Stress

## INTRODUCTION

Reactive oxygen species (ROS) are normal reactive byproducts of oxygen metabolism that were originally considered to be universally damaging cellular molecules capable of producing injurious effects on lipids, proteins, and DNA.[21] However, it is now known that ROS also play an integral role in normal cell function including cellular metabolism, signaling, and regulation.[26] Under physiologic conditions, ROS are produced within the vasculature in a controlled manner, producing low concentrations of these vital signaling molecules, which play a role in normal vascular smooth muscle cell contraction [9] and growth.[40,46,53] A pathophysiologic state ensues when there is either over-production of ROS, a diminished capacity to regulate ROS concentrations due to reduced antioxidant defense mechanisms,[16] or both.

20-Hydroxyeicosatetraenoic acid (20-HETE) is a vasoconstrictor metabolite of arachidonic acid that is formed endogenously in a number of vascular beds through the action of cytochrome P450 ω-hydroxylase (CYP) enzymes of the 4A and 4F family. [22–24,32,33] 20-HETE appears to play a crucial role in the regulation of blood flow, $O_2$ sensitivity, and vascular function.[22–24,32,33] The CYP4A/20-HETE pathway can also contribute to oxidative stress in tissues because the action of the CYP4A enzyme requires the ROS-producing enzyme NADPH oxidase as a necessary co-factor [5]. 20-HETE can also directly uncouple endothelial nitric oxide synthase (eNOS) through a signaling cascade involving tyrosine kinase stimulation of the mitogen-activated protein kinase and extracellular signal-regulated kinase pathways.[8] This signaling cascade blocks the association between eNOS and heat shock protein 90 (HSP90), which is critical for the normal function of eNOS. [7,8,50] Thus, the CYP4A/20-HETE pathway is not only involved in the direct production of ROS, but also in eNOS uncoupling, leading to reduced NO production and elevated vascular levels of superoxide anion ($O_2^-$).

The Dahl salt-sensitive (SS) rat is an inbred genetic model of salt-sensitive hypertension that is susceptible to heightened oxidative stress due, at least in part, to reduced antioxidant defense mechanisms. For example, Somova et al. [42] demonstrated decreased glutathione levels and reduced glutathione peroxidase activity (indicators of antioxidant defenses) in the myocardium of Dahl SS rats. Our laboratory and others have shown that Dahl SS rats have reduced levels of antioxidant enzymes including Cu/Zn-SOD in their cerebral vasculature, [13] as well as reduced renal medullary Cu/Zn-SOD and Mn-SOD expression.[38] Collectively, those findings are consistent with the hypothesis that Dahl SS rats have reduced antioxidant defense mechanisms, leaving them vulnerable to any mechanism that may generate reactive oxygen species.

Previous studies in our laboratory have shown that cerebral arteries from SS rats exhibit a pathological upregulation of the CYP4A/20-HETE pathway resulting in 20-HETE-dependent vascular dysfunction.[35] Mesenteric resistance arteries from Sprague-Dawley

rats fed a high salt diet also fail to dilate in response to reduced $PO_2$--a response that can be restored in the presence of the CYP4A inhibitor N-methyl-sulfonyl-12,12-dibromododec-11-enamide (DDMS).[49] The salt-induced vascular dysfunction in mesenteric resistance arteries of Sprague-Dawley rats is due in part to elevated vascular CYP4A protein expression[49] and elevated vascular ROS, as evaluated via DHE fluorescence [56]. Thus, in both Dahl SS rats and HS-fed Sprague-Dawley rats, an increase in CYP4A protein expression is observed concomitant with a highly oxidant environment suggesting that either the CYP4A/20-HETE pathway is producing reactive oxygen species or, perhaps, that ROS levels are regulating the expression of the CYP4A/20-HETE pathway.

The present study tested the hypothesis that upregulation of the CYP4A/20-HETE pathway results in cerebral vascular dysfunction in the Dahl SS rat due to the production of reactive oxygen species (ROS). We also evaluated whether elevated vascular ROS levels may upregulate the expression of the CYP4A/20-HETE pathway in the vessels. This hypothesis was tested by comparing the Dahl SS rat to its close genetic counterpart, the SS-$5^{BN}$ consomic rat; which has chromosome 5 carrying the 20-HETE-producing CYP4A alleles from the normotensive Brown Norway (BN) rat introgressed into the SS genetic background. The SS-$5^{BN}$ consomic rat is protected from salt-induced hypertension,[36,51] vascular dysfunction, and vascular oxidative stress [35] that is present in the Dahl SS rat; and these protective effects of chromosomal substitution appear to be due, at least in part, to a decreased contribution of the CYP4A/20-HETE pathway.[35]

## MATERIALS AND METHODS

### Experimental Groups

Eight to 12 week old male SS-$5^{BN}$ consomic rats (SS-Chr $5^{BN}$/Mcwi strain) and Dahl SS rats were used for this study. Following weaning, Dahl SS rats were maintained on a normal salt (0.4% NaCl) diet and then switched to a high salt (HS; 4.0% NaCl; Dyets, Inc.) diet for 3 days immediately prior to the experiments, with water to drink *ad libitum*. A subset of HS-fed Dahl SS rats were given drinking water with either the superoxide scavenger Tempol (1 mM) alone or Tempol (1mM) and the nitric oxide synthase inhibitor NG-nitro-l-arginine methyl ester (L-NAME; 40mg/kg/day) for 7 days. Previous studies have shown that this duration and dosage of Tempol does not significantly alter blood pressure, regardless of salt intake [55].

The SS-$5^{BN}$ consomic rats were divided into three treatment groups: 1) switched to a high salt diet for 3 days; 2) HS-fed SS-$5^{BN}$ consomic rats receiving an i.v. infusion of the superoxide dismutase inhibitor, diethyldithiocarbamate (DETC) at a low-dose (16 mg/kg/day) for 7 days followed by three days of high dose DETC (200 mg/kg/day); or 3) the same conditions as Group 2 with Tempol in the drinking water for 7 days immediately prior to harvesting the vessels. The animals in Groups 2 and 3 were put on a HS diet for the final 3 days of the treatment session. The Medical College of Wisconsin IACUC approved all protocols.

## Isolated Vessel Experiments

Animals were anesthetized with an intramuscular injection containing (in mg/kg): ketamine (75.0), acepromazine (2.5) and anased (10.0). Middle cerebral arteries (MCA) were isolated, cannulated with micropipettes, and perfused and superfused with bicarbonate-buffered physiological salt solution as previously described [20]. Internal diameter was measured using television microscopy, and vessels lacking active tone at rest were excluded from study.

After the control equilibration period, responses to the endothelium-dependent dilator acetylcholine (ACh; $10^{-10}$–$10^{-5}$ M) were determined. The vessels were then incubated for 30 minutes in the presence of DDMS (50 μM) to inhibit CYP4A enzymes and the responses to ACh were repeated. Time control experiments showed no effect of incubation time on vascular responses. In a second series of experiments, acute Tempol (100 μM) was added to the perfusate and superfusate for the final 30 minutes of the control equilibration period and the responses of the arteries to acetylcholine were then recorded.

Vessel responses to an NO donor were not tested in the present study, because our earlier studies [35] showed that there is no difference in the response of MCA to an exogenous nitric oxide donor in Dahl SS and SS-$5^{BN}$ rats fed either a normal salt or high salt diet; and no effect of DDMS on vessel responses to NO donors. Because DDMS lowers vascular ROS levels similar to Tempol, we believe that vessel responses to SNP would also be unaffected by Tempol treatment, although this remains to be established.

At the end of the experiment, the maximum diameter of the artery was determined by adding hydrogen peroxide ($H_2O_2$; 1.76 mM) to the superfusate to achieve maximum dilation. Active resting tone (%) was calculated as $[(D_{max}-D_{rest})/D_{max}] \times 100$, where $D_{max}$ is the maximum diameter in the presence of $H_2O_2$ and $D_{rest}$ is the resting control diameter (Table 1).

## Dihydroethidium Fluorescence

Vascular levels of reactive oxygen species (ROS) were assessed using dihydroethidium (DHE) fluorescence [13] in basilar arteries. Basilar arteries were used as a substitute for MCA, as both vessels demonstrate a NO-dependent dilation to ACh,[37] and the larger diameter of the basilar artery allows for improved cross sectioning. In those experiments, isolated arteries were incubated for 30 minutes in PSS heated to 37°C, followed by an additional 30 minute incubation with either PSS alone, DDMS (50 μM), PEG-SOD (100 U/mL), or L-NAME (100 μM). The vessels were then incubated with DHE (5 μmol/L) for an additional 15 minutes. The arteries were cut into 10 μm transverse sections and imaged with a Nikon Eclipse TS100 microscope (Nikon, Tokyo, Japan) equipped with a ×20 objective, a 540 nm excitation filter, a 605 nm emission filter (Chroma Technology Corp., Bellows Falls, VT) and QImaging Regiga-2000R digital camera (Surrey, British Columbia, Canada). Multiple images of each artery were quantified using ImageJ software and background fluorescence was subtracted from the fluorescence value of the arterial ring [13].

### Western Blot

CYP4A protein expression in cerebral arteries was assessed by Western blot using CYP4A1/A2/A3 antibody (Santa Cruz, sc-53247), as previously described.[32,48,49] 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).

### Statistical Analysis

Data were summarized as mean ± SEM. 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 analysis of variance (ANOVA), and 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

### Vessel Diameter and Active Tone in SS-5$^{BN}$ Consomic Rats and Dahl SS Rats

Maximum diameter, resting diameter, and active tone in middle cerebral arteries of SS-5$^{BN}$ consomic rats and SS rats are presented in Table 1. Active tone of isolated MCA from SS-5$^{BN}$ consomic rats was unaffected by chronic treatment with either the SOD inhibitor DETC or the SOD mimetic Tempol; and there was no effect of acute addition of either Tempol or the CYP4A inhibitor DDMS to the tissue bath. Collectively, these findings demonstrate that any differences in the magnitude of vascular relaxation responses observed in the present study were not due to initial differences in resting tone as a result of a pre-existing constriction of the artery or structural differences between treatment groups.

### Responses to Acetylcholine in Middle Cerebral Arteries of Dahl SS and SS-5$^{BN}$ Consomic Rats

Figure 1 summarizes vascular relaxation in response to the endothelium-dependent vasodilator acetylcholine (ACh) in MCA exhibiting myogenic tone from Dahl SS and SS-5$^{BN}$ consomic rats. MCA from HS-fed Dahl SS rats failed to dilate to ACh in control conditions. Vascular relaxation to ACh in MCA of SS rats was restored in the presence of either the CYP4A inhibitor DDMS or the superoxide scavenger Tempol (Figure 1A).

Arteries from HS-fed SS-5$^{BN}$ consomic rats dilated to ACh in control conditions, but ACh-induced dilation was significantly reduced when oxidative stress was artificially elevated by chronic infusion of the SOD inhibitor DETC (Figure 1B). Incubating arteries from DETC-treated SS-5$^{BN}$ rats with the CYP4A inhibitor DDMS restored ACh-induced dilation in those animals. Similar to HS-fed Dahl SS rats, ACh-induced relaxation of middle cerebral arteries from DETC-treated SS-5$^{BN}$ rats was restored either by acute incubation with Tempol, or chronic administration of Tempol in the drinking water. Addition of DDMS to the tissue bath in the presence of Tempol showed no additive effect (Figure 1C).

### Vascular Oxidative Stress in Cerebral Arteries of Dahl SS Rats

Figure 2 summarizes the ROS levels estimated semi-quantitatively via DHE fluorescence in basilar arteries from HS-fed Dahl SS rats treated with DDMS, L-NAME, or PEG-SOD. In those experiments, both DDMS and L-NAME reduced vascular ROS levels to the same degree as PEG-SOD.

### Vascular Oxidative Stress in Cerebral Arteries from SS-5$^{BN}$ Consomic Rats

ROS levels in basilar arteries from HS-fed SS-5$^{BN}$ consomic rats incubated with the CYP4A inhibitor DDMS were not significantly different from those in non-treated arteries (Figure 3), confirming that levels of ROS in these arteries were not significantly affected by the CYP4A/20-HETE pathway. Chronic treatment with the SOD inhibitor DETC elevated vascular ROS levels compared to untreated SS-5$^{BN}$ consomic rats. Importantly, incubation of basilar arteries from the DETC-treated SS-5$^{BN}$ consomic rats with DDMS restored vascular ROS levels to control values.

### CYP4A Enzyme Expression in Cerebral Arteries from Dahl SS and SS-5$^{BN}$ Consomic Rats

We have previously shown that CYP4A enzyme expression is lower in SS-5$^{BN}$ rats fed either NS or HS diet compared to SS rats on a similar diet.[35] Figure 4A summarizes the expression of CYP4A enzyme protein in cerebral arteries from HS-fed Dahl SS rats in control conditions, during chronic treatment with the SOD mimetic Tempol, or chronic co-treatment with Tempol and L-NAME. Treatment with Tempol significantly reduced CYP4A protein expression in arteries of Dahl SS rats. Co-treatment with L-NAME + Tempol prevented the reduction in CYP4A protein expression in SS rats. In DETC-treated SS-5$^{BN}$ consomic rats in which ROS levels were artificially elevated by the SOD inhibitor, chronic treatment with Tempol to scavenge superoxide reduced the expression of CYP4A enzyme proteins compared to animals that did not receive Tempol (Figure 4B).

## DISCUSSION

Reactive oxygen species and the subgroup of oxygen-derived free radicals that includes superoxide anion and hydroxyl radical, play a key role in the pathogenesis of hypertension.[2,28] Multiple animal models of salt-sensitive hypertension [Dahl SS, stroke prone spontaneously hypertensive rat, and mineralocorticoid hypertension], all share in common elevated levels of superoxide anion in either the vasculature or the kidney.[38,47] Dahl SS rats have elevated renal [38,47] and vascular [13,44] superoxide production, and also exhibit reduced renal medullary Cu/Zn-SOD and Mn-SOD expression[38] and reduced cerebral artery Cu/Zn-SOD expression.[13] Other studies [35, 36] have shown that Dahl SS rats are vulnerable to the development of vascular dysfunction due to their limited antioxidant defense mechanisms. In those studies, vascular function was restored when Cu/Zn SOD expression was upregulated in response to either chronic infusion of a low dose of angiotensin II [14] or by introgression of a normally functioning Brown Norway renin gene into the SS genetic background. [13]

A major component of the deleterious effects of ROS within the vasculature is the reaction between nitric oxide and superoxide anion, which not only reduces the bioavailability of

nitric oxide, but also produces the highly reactive nitrogen species, peroxynitrite [17]. Peroxynitrite directly affects vascular tone by inhibiting calcium-activated potassium channels [3] and alters normal cellular signaling by nitration of tyrosine and tryptophan residues [1,30]. Peroxynitrite is also a significant contributor to oxidative stress via eNOS uncoupling due to oxidation of the necessary cofactor tetrahydrobiopterin (BH4) and destruction of the heme/heme center of eNOS [6].

Previous work in our laboratory has shown that acute scavenging of superoxide anions with Tempol, which restores nitric oxide bioavailability, restores vasodilator responses to ACh and reduced $PO_2$ in cerebral arteries from SS rats. [12] Chronic treatment with Tempol in the drinking water also restores endothelium-dependent dilation to ACh in arteries of Dahl SS rats, regardless of dietary salt intake.[15] In the present study, chronic scavenging of superoxide radicals with Tempol also ameliorated the impaired relaxation in response to ACh in MCA from HS-fed SS rats, presumably by restoring NO bioavailability in the vasculature, as the protective effect of chronic Tempol treatment to restore acetylcholine-induced dilation in SS rats is eliminated by the NO synthase inhibitor L-NAME[15]. Taken together, these data are consistent with multiple reports that elevated ROS levels are a major contributor to endothelial dysfunction in the Dahl SS rat.

Previous reports have shown that basilar arteries from Dahl SS rats have elevated levels of vascular reactive oxygen species compared to SS-$5^{BN}$ consomic rats [35]. It is interesting to note that vessels from the SS-$5^{BN}$ rat, which shares ~95% genetic homology to the Dahl SS rat, exhibit no differences in expression of superoxide dismutase enzymes (Cu/Zn SOD, MnSOD, or ec-SOD) or endothelial nitric oxide synthase compared to Dahl SS rats, regardless of dietary salt intake.[35] Based on these findings, the reduced antioxidant defenses in Dahl SS rats, leaving them susceptible to vascular assault by ROS, should also be present in the SS-$5^{BN}$ rat. However, the SS-$5^{BN}$ rat demonstrates normal vascular function when fed either a normal salt (0.4% NaCl) or a high salt diet suggesting that, in addition to limited antioxidant defenses, there is an elevated production of ROS in the SS rat compared to the SS-$5^{BN}$ rat.

The CYP4A/20-HETE pathway appears to be a major contributor to elevated vascular oxidant stress in HS-fed SS rats, as arteries from HS-fed Dahl SS rats exhibit a significant reduction in vascular ROS levels following incubation with the CYP4A inhibitor DDMS. [35] Production of 20-HETE has the capacity to increase vascular ROS via two possible mechanisms: 1) by 20-HETE-induced NADPH oxidase activation [27,41,52,54] and 2) through the uncoupling of eNOS [7,8]. As noted above, 20-HETE has been shown to directly uncouple eNOS by interfering with the interaction of eNOS with HSP90. [7]

In the present study, basilar arteries from high salt-fed Dahl SS rats incubated with L-NAME to inhibit eNOS had reduced ROS accumulation to the same degree as vessels treated with either DDMS or the superoxide scavenger PEG-SOD. Based on the nearly identical responses of vascular ROS levels to DDMS and L-NAME treatment, it is reasonable to postulate that the upregulation of the CYP4A/20-HETE pathway that is present in cerebral arteries of Dahl SS rats [35] contributes to oxidative stress via uncoupling of eNOS, as vascular ROS levels were reduced to the same degree by treatment with either L-NAME or

DDMS. Taken together, these observations suggest that 20-HETE either directly uncouples eNOS [27,41,52,54] or that the oxidative environment supported by elevated 20-HETE production leads to eNOS uncoupling by another mechanism such as peroxynitrite-induced BH4 oxidation [6].

The normal dilation of the MCA in response to ACh in arteries of HS-fed SS-$5^{BN}$ consomic rats was unexpected, and contrasts with the loss of endothelium-dependent dilation in arteries of HS-fed Sprague-Dawley rats, [45] and HS-fed SS-$13^{BN}$ consomic rats, [11] and cheek pouch arterioles of HS-fed hamsters. [39] One important difference between these findings is that HS diet leads to a significant increase in vascular ROS levels in HS-fed Sprague-Dawley rats [56] and hamster arteries, [39] but not in arteries from HS-fed SS-$5^{BN}$ rats (Figure 3).

In the present study, ROS levels in basilar arteries from HS-fed SS-$5^{BN}$ consomic rats incubated with the CYP4A inhibitor DDMS were not significantly different from those in non-treated arteries (Figure 3), confirming that levels of ROS in these arteries were not significantly affected by the CYP4A/20-HETE pathway. Chronic treatment with the SOD inhibitor DETC elevated vascular ROS levels compared to untreated SS-$5^{BN}$ consomic rats. Importantly, incubation of basilar arteries from the DETC-treated SS-$5^{BN}$ consomic rats with DDMS restored vascular ROS levels to control values, supporting the concept that the CYP4A/20-HETE pathway can contribute to vascular ROS production in the cerebral circulation. Although unlikely, another possible explanation for the latter finding could be a previously unreported effect of the CYP4A inhibitor directly on vascular ROS production. In light these observations, direct measurements of 20-HETE levels in the presence and absence of DDMS would be a valuable direction for future investigation, to exclude any unforeseen off target effects of DDMS.

Taken together, the findings of the present study support the hypothesis that the lower ROS levels in the HS-fed SS-$5^{BN}$ rats are crucial to maintaining the normal endothelium-dependent relaxation of the MCA in response to ACh in these animals. Specifically, with lower superoxide levels to combine with and degrade nitric oxide, there would be improved NO bioavailability to respond appropriately to stimulation by ACh in the SS-$5^{BN}$ vasculature. While it is tempting to speculate that the lower ROS levels and maintained endothelial function in the HS-fed SS-$5^{BN}$ rats are due to the presence of the Brown Norway CYP4A alleles on chromosome 5, it is important to note that the genes coding for CYP4A alleles on chromosome 5 are not the only BN genes that may differ from those in the SS rat, which could impact the findings of the present study. Therefore, a valuable target for future investigation is to develop narrowed congenic strains that contain smaller and smaller segments of the BN chromosome 5 that either include or exclude the BN CYP4A alleles, similar to previously reported studies isolating the protective effects chromosome 13 to the presence of a normally functioning BN renin allele in narrowed congenic strains derived from SS-$13^{BN}$ consomic rats.[15]

Chronic treatment with DETC to inhibit superoxide dismutase artificially elevates reactive oxygen species in the SS-$5^{BN}$ rats (Figure 3), allowing the accumulation of superoxide to go unabated by antioxidant defense mechanisms, which would result in superoxide-induced

degradation of nitric oxide. Consistent with this hypothesis, MCA from DETC-treated SS-$5^{BN}$ consomic rats failed to dilate in response to ACh under control conditions. However, both acute and chronic treatment with Tempol to scavenge superoxide restored vascular relaxation in response to ACh in DETC-treated SS-$5^{BN}$ consomic rats. Collectively, these observations support the fundamental hypothesis of the present study, namely that, under conditions of reduced antioxidant defense mechanisms (i.e. in the Dahl SS rat), any oxidant-producing pathway contributes to the loss of vascular function. Therefore, inhibiting the CYP4A/20-HETE pathway and reducing vascular oxidative stress would restore NO-dependent dilation to ACh in arteries of the SS rats.

Previous studies in our laboratory [34] [35] have shown that ROS levels, assessed with DHE, are significantly lower in cerebral arteries of SS-$5^{BN}$ rats fed normal salt diet compared to those in SS rats fed normal salt diet. Those studies also showed that the expression of CYP4A enzyme protein, assessed by Western blotting, was also significantly higher in cerebral arteries of SS rats compared SS-$5^{BN}$ consomic rats fed either normal or high salt diet [34] [35].

In the present study, chronic antioxidant treatment with Tempol significantly reduced CYP4A protein levels in cerebral arteries from HS-fed Dahl SS rats and HS-fed SS-$5^{BN}$ rats treated with the SOD inhibitor DETC to artificially elevate superoxide levels compared to arteries from control animals. Co-treatment with L-NAME + Tempol prevented the reduction in CYP4A protein expression in SS rats, suggesting that the effect of Tempol to reduce the expression of CYP4A enzyme proteins is mediated via restoration of nitric oxide bioavailability in the presence of the superoxide scavenger. In DETC-treated SS-$5^{BN}$ consomic rats in which ROS levels were artificially elevated by the SOD inhibitor, chronic treatment with Tempol to scavenge superoxide also reduced the expression of CYP4A enzyme proteins in SS rats compared to animals that did not receive Tempol (Figure 4B), again supporting a role for ROS-dependent increases in vascular CYP4A protein expression. To our knowledge, this is the first demonstration of a regulatory effect of reactive oxygen species on the expression of CYP4A enzyme proteins.

The present findings are in contrast to reports from studies of isolated vascular smooth muscle cells [25] and renal microsomes,[29] showing that acute Tempol treatment has no effect on 20-HETE production and that acute addition of high concentrations of a superoxide donor reduces 20-HETE production. However, in those studies, the acute administration of either Tempol or the superoxide donor would not allow sufficient time to alter protein expression, as was observed in the present study; and, the use of isolated cells and prepared microsomes may not accurately reflect *in vivo* conditions. In addition, the concentration of superoxide used in those studies was likely high enough to scavenge 20-HETE via lipid peroxidation to form the more polar isoprostane [29], and to reduce the bioavailability of necessary co-factors through oxidation, which would substantially reduce 20-HETE production as well [10,26,52].

We previously reported that it is possible for reactive oxygen species to exert a regulatory influence over the CYP4A/20-HETE pathway--not directly, but as an indirect result of the effect of superoxide on nitric oxide [35]. Superoxide anion can affect basal levels of nitric

oxide because $O_2^-$ reacts with NO at a rate three times faster than its interaction with SOD. [18] In the Dahl SS rat, NO availability in the vasculature is reduced due to the elevated vascular ROS. [12,15] Nitric oxide inhibits CYP4A activity by forming a ferrous-nitrosyl complex at the heme binding site of the CYP4A enzyme, presumably reducing 20-HETE production.[43] Studies in other laboratories have shown that nitric oxide also has the capacity to suppress both mRNA and protein expression of cytochrome P450 enzymes in cultured hepatocytes and in liver microsomes, [19,31] The present studies support a role for NO in regulating not only the activity of the CYP4A enzymes, but also the expression of CYP4A protein in the vasculature.

In arteries of HS-fed Dahl SS rats, the inhibitory influence of NO on the CYP4A/20-HETE pathway would be absent due to the ROS-induced degradation of nitric oxide, providing a possible explanation for the elevated vascular CYP4A protein expression in arteries of Dahl SS rats compared to those of SS-$5^{BN}$ consomic rats.[35] Cerebral arteries from HS-fed Dahl SS rats chronically treated with a combination of Tempol to scavenge superoxide and the NOS inhibitor L-NAME to prevent the Tempol-induced restoration of NO bioavailability had elevated CYP4A protein levels similar to those of the control animals (Figure 4). These data provide compelling evidence that the ROS-induced CYP4A upregulation results indirectly from the loss of NO bioavailability and, therefore, removal of NO-induced CYP4A inhibition. This novel finding of ROS-induced regulation of CYP4A protein expression occurred in both the Dahl SS rat and in the SS-$5^{BN}$ strain, supporting our hypothesis that oxidative stress directly contributes to the upregulation of the CYP4A/20-HETE pathway.

A situation where reactive oxygen species stimulate a ROS-producing pathway (the CYP4A/20-HETE pathway) sets the stage for a potentially catastrophic exacerbation of ROS accumulation, especially in the presence of limited antioxidant defenses, as present in the Dahl SS rat.[13] Taking into consideration the necessity of ROS signaling for normal cell survival,[26] it is clear that a reduction in antioxidant defense mechanisms could result in the pathological oxidant environments observed in hypertensive individuals and patients with many other cardiovascular diseases[4,28]. A positive feedback loop involving the CYP4A/20-HETE pathway provides one possible explanation for the spiraling effects of increased ROS production into an advanced disease state, such as hypertension, atherosclerosis, and other cardiovascular diseases characterized by endothelial dysfunction.

## Perspectives

This study used a novel consomic rat strain (the SS-$5^{BN}$ consomic rat) which is identical to the Dahl salt-sensitive (SS) rat genetically except for the introgression of Brown Norway chromosome 5 carrying the BN cytochrome P450-4A (CYP4A) alleles into the Dahl SS genetic background. The results of this study demonstrate not only a role for the CYP4A/20-HETE pathway in producing reactive oxygen species (ROS) (as CYP4A inhibition reduced vascular oxidative stress); but also a regulatory influence of vascular free radicals on CYP4A protein expression—most likely as a result of reduced nitric oxide bioavailability (as manipulation of local ROS levels altered CYP4A protein expression and the beneficial effect of CYP4A enzyme inhibition on vascular CYP4A enzyme expression was prevented by

inhibiting NOS with L-NAME). This potential positive feedback loop between increased CYP4A enzyme expression/activity, elevated vascular ROS, reduced NO availability, and elimination of NO-mediated downregulation of the CYP4A/20-HETE system could play a major role in the exacerbation of cardiovascular diseases characterized by elevated levels of vascular oxidant stress in humans.

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## Abbreviations

| | |
|---|---|
| **ACh** | Acetylcholine |
| **ANOVA** | Analysis of Variance |
| **BH4** | Tetrahydrobiopterin |
| **BN** | Brown Norway |
| **Cu/Zn-SOD** | Copper/Zinc Superoxide Dismutase (SOD 1) |
| **CYP** | Cytochrome P450 |
| **CYP4A** | Cytochrome P450-4A ω-Hydroxylase |
| **DDMS** | N-methyl-sulfonyl-12,12-dibromododec-11-enamide |
| **DETC** | Diethyldithiocarbamate |
| **DHE** | Dihydroethidium |
| **eNOS** | Endothelial Nitric Oxide Synthase (NOS 3) |
| **HSP90** | Heat Shock Protein 90 |
| **L-NAME** | NG-nitro-l-arginine methyl ester |
| **Mn-SOD** | Manganese Superoxide Dismutase (SOD 2) |
| **PEG-SOD** | Polyethylene Glycol Superoxide Dismutase |
| **ROS** | Reactive Oxygen Species |
| **SOD** | Superoxide Dismutase |
| **SS rats** | Dahl Salt-Sensitive Rats |
| **SS-5$^{BN}$** | SS-5$^{BN}$ Consomic Rats (SS-Chr 5$^{BN}$/Mcwi strain) |
| **SS-13$^{BN}$** | SS-13$^{BN}$ Consomic Rats |

**20-HETE** 20-Hydroxyeicosatetraenoic acid

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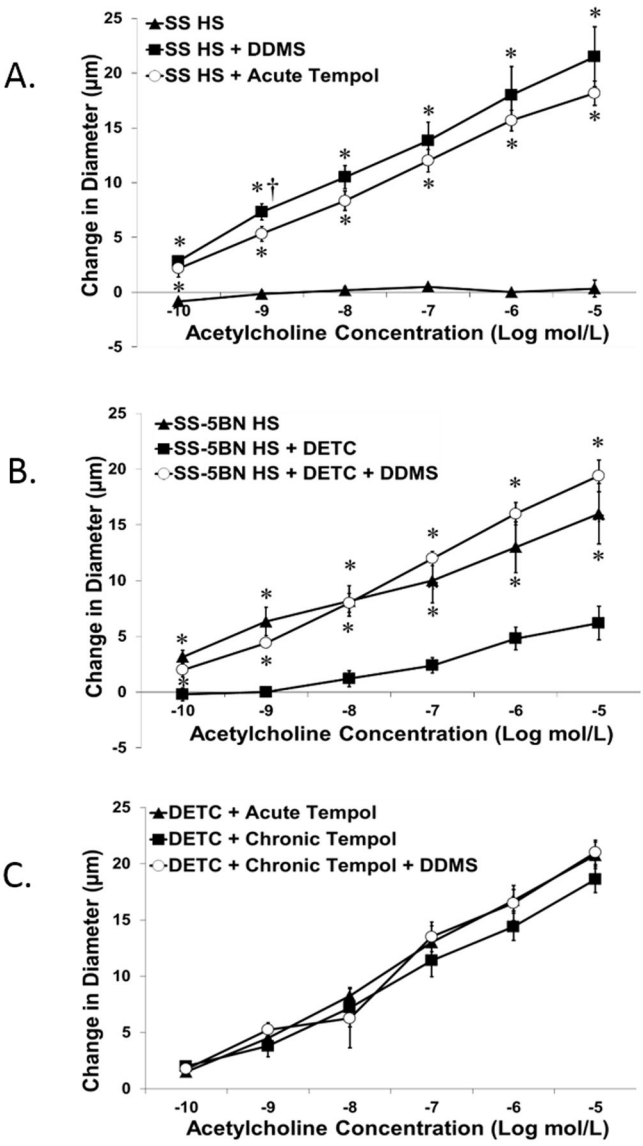



**Figure 1.**

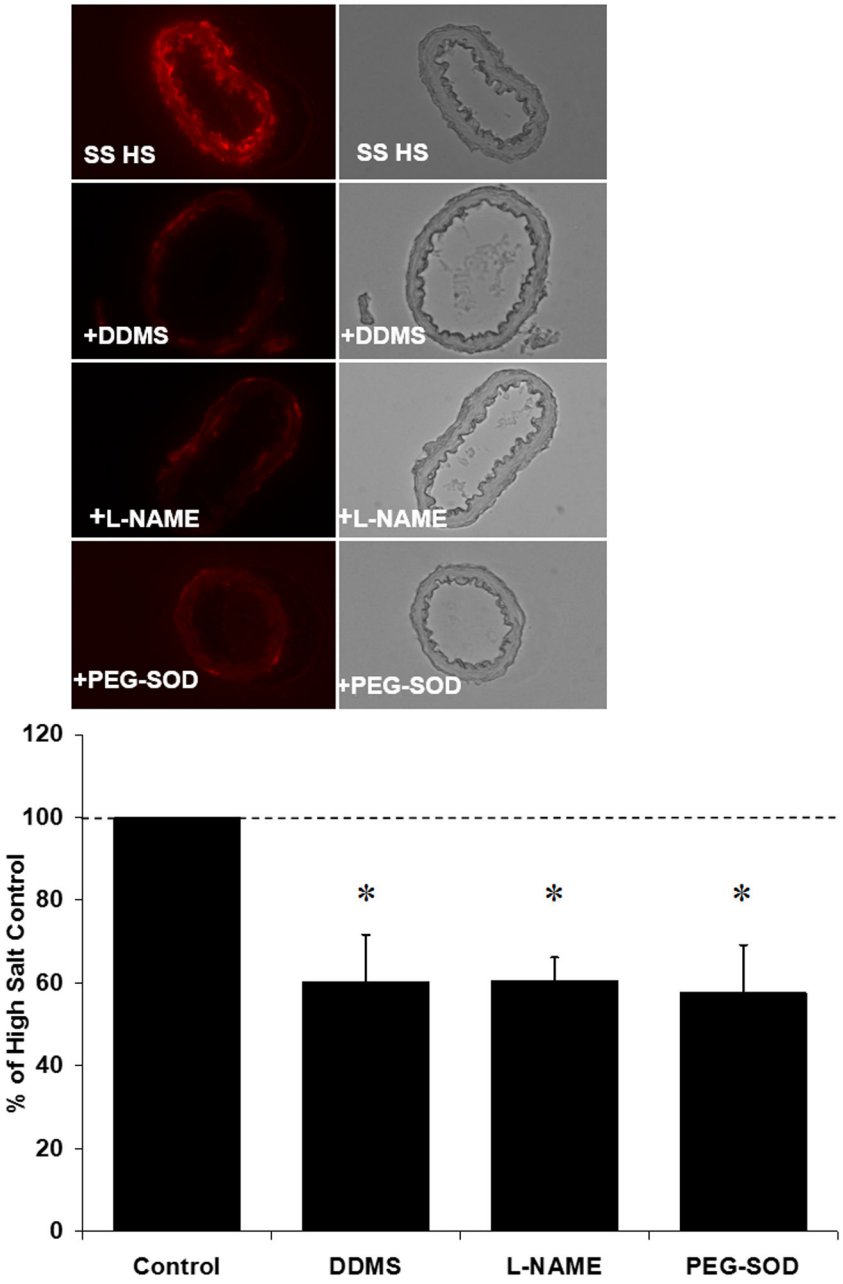
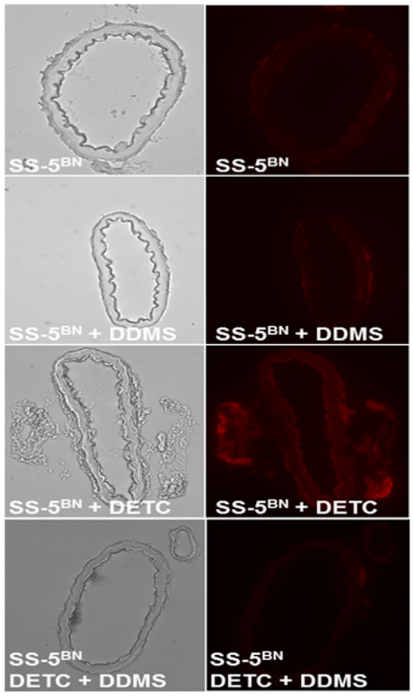



**Figure 2.**



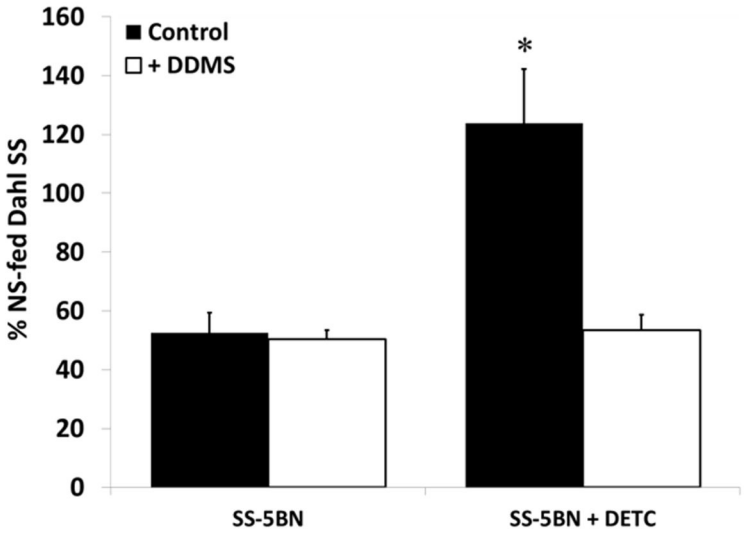




**Figure 3.**

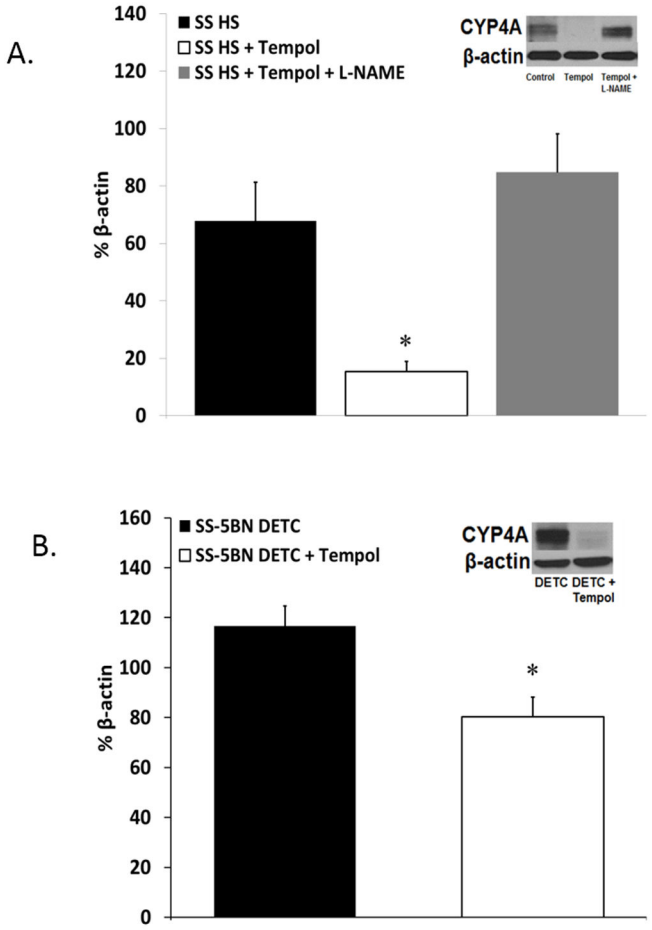



**Figure 4.**

## Table 1

Middle Cerebral Artery Diameters and Resting Tone

| Treatment Groups | n | Resting Diameter (μm) | Maximum Diameter (μm) | % Active Tone |
|---|---|---|---|---|
| **SS HS** | 6 | 143±11 | 239±5.1 | 40±5.2 |
| **SS HS + DDMS** | 5 | 127±7.4 | 236±1.8 | 46±3.2 |
| **SS HS + Acute Tempol** | 6 | 135±8.2 | 230±2.8 | 41±3.8 |
| **SS-5$^{BN}$ HS** | 6 | 128±6.3 | 242±3.9 | 47±3.2 |
| **DETC-treated SS-5$^{BN}$** | 5 | 132±6.8 | 242±3.5 | 45±3.1 |
| **DETC-treated SS-5$^{BN}$ + Acute Tempol** | 4 | 122±6.7 | 242±4.5 | 50±2.7 |
| **DETC-treated SS-5$^{BN}$ + DDMS** | 5 | 152±8.4 | 243±2.9 | 37±3.7 |
| **DETC-treated SS-5$^{BN}$ + Chronic Tempol** | 5 | 124±6.3 | 239±3.2 | 48±2.6 |
| **DETC-treated SS-5$^{BN}$ + Chronic Tempol + DDMS** | 4 | 131±18.6 | 239±3.9 | 45±8.4 |