Role of the CYP4A/20-HETE Pathway in Vascular Dysfunction of the Dahl Salt-sensitive Rat

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Abstract
20-HETE (20-hydroxyeicosatetraenoic acid), a vasoconstrictor metabolite of arachidonic acid formed through the action of CYP4A (cytochrome P450-4A) in vascular smooth muscle cells, has been implicated in the development of hypertension and vascular dysfunction. There have been a number of reports in human subjects demonstrating an association between elevated urinary excretion of 20-HETE and hypertension, as well as increased 20-HETE production and vascular dysfunction. The Dahl SS (salt-sensitive) rat is a genetic model of salt-sensitive hypertension that exhibits vascular dysfunction, even when maintained on a normal-salt diet and before the development of hypertension. This mini-review highlights our current research on the role of CYP4A and 20-HETE in the vascular dysfunction of the Dahl SS rat. In our studies, the SS rat is compared with the consomic SS-5BN rat, having chromosome 5 from the salt-resistant Brown Norway rat (carrying all CYP4A genes) introgressed on to the SS genetic background. Our laboratory has demonstrated restoration of normal vascular function in the SS rat with inhibition of the CYP4A/20-HETE pathway, suggesting a direct role for this pathway in the vascular dysfunction in this animal model. Our studies have also shown that the SS rat has an up-regulated CYP4A/20-HETE pathway within their cerebral vasculature compared with the SS-5BN consomic rat, which causes endothelial dysfunction through the production of ROS (reactive oxygen species). Our data shows that ROS influences the expression of the CYP4A/20-HETE pathway in the SS rat in a feed-forward mechanism whereby elevated ROS stimulates production of 20-HETE. The presence of this vicious cycle offers a possible explanation for the spiralling effects of elevated 20-HETE on the development of vascular dysfunction in this animal model.

Key words: cerebral circulation, cytochrome P450-4A (CYP4A), 20-hydroxyeicosatetraenoic acid (20-HETE), oxidative stress, salt-sensitive, vascular function

20-HETE (20-hydroxyeicosatetraenoic acid), a vasoconstrictor metabolite of arachidonic acid generated by CYP4A (cytochrome P450 4A) enzymes, plays a vital role in vascular function and the onset and progression of cardiovascular disease [1]. Studies on human subjects have shown an association between genetic variants in the human gene that forms 20-HETE and an increase in both mean arterial pressure and 20-HETE production [2,3]. In animal models, SHRs (spontaneously hypertensive rats) [4] and androgen-induced hypertensive rats [5] have elevated vascular 20-HETE and high BP (blood pressure), which can be reversed with inhibition of CYP4A.

Our laboratory has shown that S–D (Sprague–Dawley) rats develop vascular dysfunction and up-regulate both CYP4A mRNA transcripts and protein expression in mesenteric resistance arteries when dietary sodium is increased [6]. Decreasing the vascular production of 20-HETE through CYP4A inhibition restores the impaired vascular relaxation in arteries from high-salt-fed S–D rats [6]. Although it is clear that the CYP4A/20-HETE system is activated in high-salt-fed S–D rats, it is not known how this system contributes to vascular dysfunction in salt-sensitive hypertension. Previous reports have demonstrated an association between functional variants of CYP4A genes and alterations in 20-HETE production in both human and rodent models of salt-sensitive hypertension [7–9].

Dahl SS (salt-sensitive) rats, an inbred genetic model of salt-sensitive hypertension, have both elevated BP in response to salt,
and severe endothelial dysfunction [10–12]. Similar to genetically predisposed salt-sensitive humans [13,14], Dahl SS rats have impaired vascular relaxation in response to multiple vasodilator stimuli accompanied by reduced NO levels and elevated superoxide levels, even when maintained on a normal salt diet and preceding the development of hypertension [10,15–18]. Dahl SS rats also have potentiated vasoconstrictor responses to elevated PO2 (partial pressure of O2) in cremasteric arterioles, a response that can be ameliorated with CYP4A inhibition [19]. Our research is focused on whether the CYP4A/20-HETE pathway plays a role in the vascular changes preceding the development of hypertension in the Dahl SS rat and what effect, if any, elevated dietary sodium has on the expression and activity of this pathway.

The Program for Genomic Applications group at the Medical College of Wisconsin substituted single chromosomes from the BN (Brown Norway) rat and introgressed them on to the genetic background of the Dahl SS rat using marker-assisted selection to create a panel of consomic rats [20]. The use of consomic rat models is an extremely beneficial tool, as it provides an experimental animal with nearly identical genetic homology compared with the SS-5BN consomic rat, carrying the CYP4A genes on chromosome 5 from the BN rat. The SS-5BN consomic rat can provide valuable information on the role of CYP4A and 20-HETE in vascular dysfunction in the Dahl SS rat because the SS-5BN consomic rat has ~95% genetic homology with the Dahl SS rat, but has a reduced pressor response to elevated dietary salt and normal vascular responses to elevated PO2 [21].

Our general hypothesis is that an up-regulation of vascular CYP4A ω-hydroxylase and subsequent overproduction of 20-HETE results in increased superoxide, reduced NO bioavailability and impaired vascular relaxation in Dahl SS rats regardless of dietary salt intake. We hypothesize further that the SS-5BN consomic rats will show protection from endothelial dysfunction due to reduced CYP4A expression and subsequently reduced 20-HETE production, causing diminished vascular oxidative stress.

In our experiments [22], male SS (JrHsd/Mcwi) and SS-5BN (Jr-Chr 5BN/Mcwi) rats at 8–10 weeks of age were fed on either an NS (normal-salt) diet (0.4% NaCl; Dyets) from weaning or switched to a HS (high-salt) diet (4.0% NaCl; Dyets) for 3 days prior to experiments, with water ad libitum. The Medical College of Wisconsin IACUC-approved all protocols. On the day of the experiment, animals were anaesthetized and MCAs (middle cerebral arteries) were isolated and cannulated with glass micropipettes. Intravascular pressure was maintained at 80 mmHg and the vessels were perfused and superfused with physiological salt solution maintained at 37°C and equilibrated with a 21% O2/5% CO2/74% N2 gas mixture. Internal diameter was measured using television microscopy and a video micrometer (model IV-550; FOR-A). Vessels that did not show active tone at rest were not used in the study. Vessels with active tone were exposed to the endothelium-dependent dilator ACh (acetylcholine) and the endothelium-independent dilator SNP (sodium nitroprusside) before and after inhibition of CYP4A with 50 μM DDMS (N-methylsulfonyl-12,12-dibromododec-11-enamide) and antagonism of the action of 20-HETE with 1 μM 20-HEDE [20-hydroxyeicosa-6(Z),15(Z)-dienoic acid]. Figure 1 depicts the chemical structure of 20-HETE along with the structure of both DDMS and 20-HEDE.

Differences in the expression of the CYP4A/20-HETE pathway were assessed by Western blotting with an antibody raised against CYP4A1, CYP4A2 and CYP4A3 (sc-53247; Santa Cruz Biotechnology), as described previously by our laboratory [6,23,24]. Relative intensity of the bands was quantified and normalized to a loading control (β-actin; A5441; Sigma) using a computer-based densitometer system and Image-Quant software (Molecular Dynamics). 20-HETE levels were assessed using LC (liquid chromatography)–MS following cerebral vessel homogenization. 20-HETE was extracted from the homogenate using ethyl acetate, and the organic layer was dried under nitrogen. Samples were reconstituted in methanol [25].

Vascular oxidative stress was assessed semi-quantitatively in basilar arteries using DHE (dihydroethidium) fluorescence, as described previously [16]. The basilar artery was used as a surrogate vessel for the MCA because of the reduced mechanical stress that occurs during removal of the vessel from the brain and the slightly larger diameter, which allows improved cross-sectioning of the artery. The basilar artery is an appropriate substitute for the MCA as both arteries demonstrate a NO-dependent dilation to ACh [26].

On the day of the experiment, basilar arteries were isolated and incubated for 1 h in PSS (physiological salt solution) heated to 37°C and then treated with untreated PSS, a CYP4A inhibitor (DDMS; 50 μM), a NOS (NO synthase) inhibitor [L-N-NAME (Nω-nitro-L-arginine methyl ester); 100 μM] or PEG [poly(ethylene)glycol]–SOD (superoxide dismutase) (100 units/ml). The arteries were then incubated with 10 μM DHE for 15 min, 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 a QImaging Regiga-2000R.
digital camera. Multiple images of each artery were taken and quantified using ImageJ software. The amount of fluorescence in each basilar artery ring was quantified by subtracting the background fluorescence of each image from the brightness value of the free-hand selected ring section, as described previously [16].

From these preparations, we observed MCAs from Dahl SS rats, fed on either the NS or HS diet, failed to respond to ACh. Vascular relaxation to ACh in cerebral resistance arteries of Dahl SS rats fed on either the NS or HS diet was restored by blocking the CYP4A system with the specific inhibitor DDMS, directly antagonizing the action of 20-HETE with 20-HEDE, and by inhibition of the CYP4A alleles from the normotensive BN rat into the SS genetic background (SS-5BN consomic rat). There was no impairment in the vascular relaxation to an exogenous NO donor SNP in NS or HS-fed Dahl SS rats, and inhibition of CYP4A did not alter the vascular smooth muscle cell response to the NO donor. Taken together, these findings suggest that the CYP4A/20-HETE pathway plays a direct role in the impaired vascular response to endothelium-dependent vasodilators in Dahl SS rats.

In our study [22], the failure of MCAs from Dahl SS rats to dilate in response to ACh was due to a reduced bioavailability of NO, most probably due to the uncoupling of eNOS (endothelial NOS). This would be consistent with the capacity of 20-HETE to interrupt the normal function of eNOS by blocking the association of the enzyme with HSP90 (heat-shock protein 90) [27,28]. Lacking this critical binding, eNOS becomes uncoupled and produces the superoxide anion instead of NO [28,29]. In our vascular preparation, inhibition of 20-HETE production with DDMS improved vascular responses to ACh, presumably by restoring the normal function of eNOS and/or normal availability of NO to dilate the vessel. The ACh-induced dilation in the presence of DDMS was eliminated by inhibiting eNOS with l-NAME and was unaffected by inhibitors of either the cyclo-oxygenase or epoxygenase pathways, demonstrating further that vascular relaxation in response to ACh depends upon a fully functional eNOS enzyme.

The difference in vascular responses between the Dahl SS and SS-5BN consomic rats appears to be due to an alteration in the CYP4A/20-HETE system. The Dahl SS rats have significantly elevated CYP4A protein expression in their cerebral vessels compared with the consomic animals on either diet. Interestingly, the differences in CYP4A protein appear to be strain-dependent only and are not influenced by dietary salt. This may be of particular importance to the Dahl SS rat, an animal model of human salt-sensitive hypertension that is predisposed to vascular dysfunction even without salt and before an elevation in arterial BP. Similarly, the ability of cerebral vessels to produce 20-HETE in the Dahl SS rat was unaffected by dietary salt intake, matching the protein expression measured in this vascular bed.

Dahl SS rats have elevated CYP4A protein expression and increased 20-HETE production, but the question as to how these elevations contribute to endothelial dysfunction remains. ROS (reactive oxygen species) and the subgroup of oxygen-derived free radicals, including superoxide anion and hydroxyl radical, play a key role in the pathogenesis of hypertension [30,31]. Multiple animal models representing salt-sensitive hypertension (Dahl SS rats, stroke-prone spontaneously hypertensive rats and mineralocorticoid hypertension), all share in common an elevated production of superoxide anion in either the vasculature or the kidney [32,33]. Dahl SS rats have both elevated renal [32,33] and vascular [16,34] superoxide production, but also exhibit reduced renal medullary Cu/Zn-SOD (copper/zinc SOD) and Mn-SOD (manganese SOD) expression [32] and reduced cerebral artery Cu/Zn-SOD expression [16].

Previous studies from our laboratory have shown that ROS are important contributors to the vascular dysfunction in the Dahl SS rat [10,35]. MCAs from Dahl SS rats treated chronically with the SOD mimetic tempol in the drinking water exhibit restored vascular relaxation in response to ACh and reduced Po2 regardless of dietary salt intake [10,35]. These data indicate that ROS contribute to endothelial dysfunction in the Dahl SS rat fed on either an NS or HS diet.

In our studies, basilar arteries from HS-fed and NS-fed Dahl SS rats had elevated vascular superoxide levels compared with SS-5BN consomic rats on either diet. It is important to note that our measurements are semi-quantitative and the following conclusions will be greatly strengthened when these studies can be repeated utilizing a quantitative method. The reduced oxidative stress in basilar arteries from the SS-5BN consomic rats undoubtedly contributes to the normal vascular responses to ACh observed in MCAs from these animals fed on either an NS or HS diet. Specifically, with reduced superoxide to combine with and degrade NO, there would be improved NO bioavailability to respond appropriately to endothelium-dependent vasodilator stimuli in the SS-5BN vasculature. Incubating basilar arteries from SS-5BN consomic rats with the CYP4A inhibitor DDMS did not reduce superoxide levels further, additionally demonstrating the lack of involvement of the CYP4A/20-HETE pathway in both vascular function and oxidant health in this close genetic counterpart to the Dahl SS rat.

The difference in vascular oxidative stress in HS-fed Dahl SS rats appears to be a direct result of the CYP4A/20-HETE pathway, as arteries from HS-fed Dahl SS rats have a significant reduction in vascular ROS levels following incubation with the CYP4A inhibitor DDMS. The production of 20-HETE has the capacity to increase vascular ROS through the catalytic process of 20-HETE formation [36–38], 20-HETE-induced NADPH oxidase activation [5,39–41] and through the uncoupling of eNOS [28,42]. Basilar arteries from HS-fed Dahl SS rats incubated with an inhibitor of eNOS (l-NAME) exhibit reduced ROS accumulation to the same degree as both DDMS-treated vessels and basilar arteries treated with the superoxide scavenger PEG–SOD. Taken together, it appears that 20-HETE may contribute to vascular oxidant stress and endothelial dysfunction in Dahl SS rats via the direct uncoupling of eNOS.

An HS diet stimulates ROS production and HS-induced AngII (angiotensin II) suppression results in lowered Cu/Zn-SOD expression in both the S–D and Dahl SS rat [16,43], thereby compromising the antioxidant defence mechanisms and further elevating ROS levels. The elevation in vascular ROS in the Dahl SS rat is concomitant with the elevated expression of CYP4A protein. Similarly, when oxidative stress was reduced via
chromosome substitution in the SS-5HN consomic rat, a close genetic counterpart to the Dahl SS rat, there was also a significant reduction in CYP4A protein. For these reasons, the possibility cannot be ignored that the elevated CYP4A protein may be contributing to ROS generation and that the accumulation of ROS could be activating the CYP4A/20-HETE pathway.

To investigate this possibility, we chronically treated Dahl SS rats with the SOD mimetic tempol and observed reduced CYP4A protein expression in cerebral arteries compared with arteries from control animals fed on the same diet [16]. This is the first time it has been reported that treatment to lower oxidative stress results in reduced CYP4A protein expression. The reduction in CYP4A protein expression in cerebral arteries from tempol-treated Dahl SS rats fed on either an NS or HS diet suggests that increased oxidative stress is contributing to the elevation in CYP4A protein in a feed-forward fashion. A situation where ROS stimulate a ROS-producing pathway (the CYP4A/20-HETE pathway) allows for a potentially catastrophic exacerbation of ROS accumulation, especially in the presence of the limited antioxidant defenses. The positive-feedback loop of the CYP4A/20-HETE pathway provides one possible explanation for the spiralling effects of increased ROS production into an advanced disease state, such as hypertension, atherosclerosis, and other cardiovascular diseases characterized by endothelial dysfunction.

Our studies demonstrate that chronic activation of the CYP4A/20-HETE pathway, as observed in cerebral arteries in the Dahl SS rat, increases vascular ROS. This presumably occurs via the direct uncoupling of eNOS, because inhibition of eNOS with l-NAME reduced ROS levels in the basilar arteries from HS-fed Dahl SS rats to the same extent as either the CYP4A inhibitor DDMS or the superoxide scavenger PEG–SOD. 20-HETE has been shown to cause eNOS uncoupling by interrupting the association between eNOS and HSP90 via NF-κB (nuclear factor-κB) activation, leading to the production of superoxide instead of NO [28,42]. In this manner, elevated 20-HETE production reduces vascular relaxation in response to vasodilator stimuli by reducing the bioavailability of NO via both diminished production of NO and elevated degradation of NO. The elevated superoxide levels existing under these circumstances would rapidly react with NO to form peroxynitrite, further reducing NO levels and compromising the ability of NO to effectively regulate vascular resistance in response to vasodilator stimuli.

The association between increased CYP4A activity and oxidative stress has also been shown in human subjects with hypertension. Ward et al. [44] have demonstrated that increased urinary 20-HETE excretion correlated positively with markers of oxidative stress, including F_{2}-isoprostanes and γ-GT (γ-glutamyl transpeptidase) and with elevated BP. Similarly, patients recovering from acute ischemic stroke have increased plasma 20-HETE concentrations and elevated plasma F_{2}-isoprostane levels compared with healthy controls, again demonstrating a relationship between increased 20-HETE production and oxidative stress [45]. Toth et al. [25] demonstrated for the first time in human cerebral arteries that 20-HETE has the capacity to produce ROS in response to flow- and pressure-induced stimuli. In fact, those authors [25] demonstrated the necessity of ROS production for effective autoregulation by 20-HETE. Thus it appears that the production of ROS by a normally functioning CYP4A/20-HETE pathway is not pathological. However, with chronic activation of this system in an animal model with reduced antioxidant defenses (or in human counterparts to this condition), 20-HETE production becomes deleterious to vascular function.

Our studies support a direct role for elevated CYP4A protein and a subsequent increase in 20-HETE production in vascular dysfunction in the Dahl SS rat. Up-regulation of CYP4A and 20-HETE is the result of a genetic alteration, as substitution of the BN genetic material for this pathway (in the SS-5HN consomic rat) ameliorates the vascular dysfunction and the overactivation of CYP4A. Although other animal models, such as the S–D rat, demonstrate salt-induced alterations to this pathway, CYP4A protein expression in the Dahl SS rat appears to be regulated, instead, by the levels of ROS in the vasculature. As summarized in Figure 2, this elevation in ROS would stimulate CYP4A protein expression in a feed-forward manner, generating excess 20-HETE, which would, in turn, produce additional ROS via uncoupling of eNOS. In the presence of the weakened antioxidant defenses of the Dahl SS rat, this vicious cycle probably results in a catastrophic deterioration of NO production and the development of vascular dysfunction. In this manner, 20-HETE could contribute to the increase in total peripheral resistance by reducing the vasculature’s ability to respond to vasodilator stimuli, directly contributing to the increase in mean arterial pressure observed in the Dahl SS rat.

**REFERENCES**

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