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Modulation by Cytochrome P450-4A ω -Hydroxylase Enzymes of Adrenergic Vasoconstriction and Response to Reduced PO₂ in Mesenteric Resistance Arteries of Dahl Salt-Sensitive Rats

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Abstract

Objective—This study evaluated the contribution of the 20-HETE/cytochrome P450-4A ω -hydroxylase (CYP4A) system to the early development of salt-induced vascular changes in Dahl salt-sensitive (SS) rats.

Methods—CYP4A expression and 20-HETE production were evaluated and responses to norepinephrine, endothelin, and reduced PO₂ were determined by video microscopy in isolated mesenteric resistance arteries from SS rats fed high salt (HS; 4% NaCl) diet for 3 days vs. low salt (LS; 0.4% NaCl) controls.

Results—CYP4A enzyme inhibition with dibromododecyl methylsulfide (DDMS) selectively reduced norepinephrine sensitivity and restored impaired vasodilation in response to reduced PO₂ in SS rats fed HS diet. In the presence of DDMS, vasodilation to reduced PO₂ was eliminated by indomethacin and unaffected by L-NAME in rats fed LS diet, and eliminated by L-NAME and unaffected by indomethacin in rats fed HS diet. The 20-HETE agonist WIT003 restored norepinephrine sensitivity in DDMS-treated arteries of HS fed rats. HS diet increased vascular 20-HETE production and CYP4A protein levels by ~24% and ~31% respectively, although these differences were not significant.

Conclusions—These findings support the hypothesis that the 20-HETE/CYP4A system modulates vessel responses to norepinephrine and vascular relaxation to reduced PO₂ in mesenteric resistance arteries of SS rats fed HS diet.

Keywords

Dahl SS rats; salt sensitive hypertension; 20-HETE; oxygen; vasodilation; vasoconstriction; cytochrome P450 ω -hydroxylase

INTRODUCTION

Salt loading leads to a reduction in the endothelium-dependent dilation in healthy human volunteers (45) and reduced salt intake improves flow-mediated vasodilation in human subjects, independent of any effect on blood pressure (4). Long-term follow-up studies in humans have shown that mortality rates are significantly higher in individuals exhibiting salt sensitivity of blood pressure, even if they fail to develop hypertension; and that the development of hypertension in the salt-sensitive individuals leads to a further increase in long-term mortality (54). All these findings are consistent with extensive evidence that endothelial dysfunction is an indicator of adverse cardiovascular events, including death, even in the absence of hypertension (55).

Vascular responses to changes in oxygen availability and to vasodilator stimuli are significantly altered in different models of hypertension including the Dahl salt-sensitive (SS) rat (40), the spontaneously hypertensive rat (SHR) (27,28,46), and rats with experimentally-induced reduced renal mass (RRM) salt-loaded hypertension (25,29). There is also evidence that increases in dietary salt intake alone impair the relaxation of rat skeletal muscle resistance arteries (12,25), pial arterioles (26), middle cerebral arteries (32), and mesenteric resistance arteries (59) in response to vasodilator stimuli such as acetylcholine, prostacyclin, and reduced PO₂, in the absence of an elevated blood pressure.

Nonetheless, the effect of high salt (HS) diet *per se* on vasoconstrictor or pressor responses in normotensive and hypertensive subjects is incompletely understood. For example, there are conflicting reports as to whether HS diet affects vascular responses to norepinephrine (2,16,18,37,38,42,53) or angiotensin II (16,34,53) in salt-sensitive and salt-insensitive experimental models. This is an important question, because any increase in the sensitivity to vasoconstrictor stimuli may aggravate the consequences of endothelial dysfunction and impaired vascular relaxation, contributing to an elevation of total peripheral resistance and an increase in arterial blood pressure.

Cytochrome P450-4A ω -hydroxylase (CYP4A) is expressed in both the cremaster muscle microcirculation and the mesenteric vascular bed of normotensive Sprague-Dawley rats (20,47,48). The production of 20-HETE by CYP4A enzymes increases as PO₂ is elevated through the physiological range (14). Consistent with the O₂ dependence of 20-HETE formation, CYP4A enzymes have been implicated in vascular O₂ sensing both in the skeletal muscle microcirculation (14,20,30) and, to a lesser extent, in skeletal muscle resistance arteries (10). In Sprague-Dawley rats, blockade of 20-HETE synthesis by inhibition of CYP4A enzymes not only reduces the sensitivity of mesenteric resistance arteries to the vasoconstrictor effects of norepinephrine (48), but also inhibits the constriction of cremasteric arterioles in response to elevated PO₂ (20,34).

Injection of antisense oligonucleotides for CYP-4A1 and -4A2 enzymes leads to a significant reduction in arterial blood pressure in Sprague-Dawley rats (51). In the SS rat model of salt-sensitive hypertension in humans, replacement of the SS alleles for cytochrome P450 isoforms by introgression of CYP4A alleles from the Lewis rat into the SS genetic background reduces the salt sensitivity of blood pressure in the resulting congenic rats (41). There is also good evidence that CYP4A enzymes and 20-HETE play a role in altered vascular reactivity in microvessels and resistance arteries in some forms of hypertension (9,19,21,49,58). However, the relative contribution of 20-HETE to vascular regulation may differ with different stages of hypertension and in different models of hypertension (19,21).

In SHR, increased 20-HETE production in mesenteric arteries is accompanied by an increased sensitivity to phenylephrine compared to normotensive Wistar-Kyoto (WKY)

controls (58). Inhibition of 20-HETE synthesis by DDMS reduces vasoconstrictor sensitivity to phenylephrine and to norepinephrine in mesenteric arteries of both SHR and WKY rats (58), and exogenous addition of 20-HETE increases phenylephrine sensitivity in DDMS-treated mesenteric resistance arteries (58). In addition, 20-HETE formation by CYP4A enzymes increases with age in Sprague-Dawley rats, contributing to age-dependent alterations in vascular regulation and having possible implications for age-related cardiovascular disease in humans (1).

The expression of some CYP4A isoforms is increased by HS diet in mesenteric arteries of Sprague-Dawley rats (48) and skeletal muscle microvessels of SS rats (49). Cremaster muscle arterioles in rats with volume expanded hypertension due to RRM show an improvement of endothelium-dependent dilation and reduced vasoconstrictor responses to angiotensin II and elevated PO_2 following inhibition of 20-HETE synthesis (9). Although all these observations suggest that salt-induced changes in the expression and activity of CYP4A enzymes are likely to contribute to alterations in vascular regulation, the exact role of the CYP4A system in contributing to vascular dysfunction and to the elevation of blood pressure in salt-sensitive hypertension, particularly in the earliest stages of the disease, is poorly understood.

The goal of the present study was to evaluate the contribution of the 20-HETE/CYP4A system to early changes in the responses of mesenteric resistance arteries to vasoconstrictors and reduced PO_2 in SS rats during short term (3 days) elevated dietary salt intake. We also determined whether the expression of CYP4A enzymes or the ability of the vessels to produce 20-HETE was altered by short-term HS diet in mesenteric resistance arteries of SS rats.

MATERIALS AND METHODS

Animal Preparation

Experiments were performed on 8-10 week-old male Dahl salt-sensitive (SS) rats (Dahl-SS/JrHsd/Mcwi). The rats were housed in the Biomedical Resource Center at the Medical College of Wisconsin (MCW), which is accredited by the American Association for the Accreditation of Laboratory Animal Care. All experimental protocols were approved by the MCW Institutional Animal Care and Use Committee. All the animals were initially fed a low salt (LS; 0.4% NaCl) AIN 76 diet (Dyets, Inc., Bethlehem, PA) with tap water *ad libitum*. Three days before the experiment, half of the rats (n=72) were switched to a high salt (HS) diet containing 4% NaCl (Dyets, Inc.), while the other half (n=74) were maintained on the low salt diet. On the day of the experiment, the rats were anesthetized with sodium pentobarbital (30 mg/kg. i.p.). A low dose of pentobarbital, sufficient to eliminate the pedal reflex, was used because SS rats have an increased sensitivity to barbiturates (43). Arterial blood pressure was measured by direct cannulation of the carotid artery of the anesthetized animals immediately prior to isolation of the small mesenteric arteries used for the vessel diameter measurements.

Evaluation of Mesenteric Vascular Reactivity to Reduced PO_2

Mesenteric resistance arteries (250–350 μ m external diameter) feeding the small intestine were isolated and cannulated with tapered glass micropipettes (with ~200 μ m tip diameter) in a tissue chamber, using standard procedures described previously (8,25,32). Arteries were incubated at 37°C and 60 mmHg intraluminal pressure for 30 minutes while continually perfusing and superfusing the vessel with physiological salt solution (PSS) equilibrated with a 21% O_2 , 5% CO_2 , 74% N_2 gas mixture. Internal vessel diameters were measured using video microscopy techniques (8,32). The PSS used in these experiments had the following

composition (in mM): 119 NaCl, 4.7 KCl, 1.17 MgSO₄, 1.6 CaCl₂, 1.18 NaH₂PO₄, 24 NaHCO₃, and 0.03 Na₂-EDTA. Arteries were pre-constricted by approximately 50% of the resting control diameter by adding 1-4 μ M norepinephrine to the vessel chamber under control (21% O₂) conditions, and changes of internal vessel diameters were measured. The O₂ concentration of the PSS in the tissue bath (superfusate) and the inflow reservoir (luminal perfusate) were then decreased by simultaneously equilibrating the PSS with a 0% O₂, 5% CO₂, 95% N₂ gas mixture, which reduces perfusate and superfusate PO₂ to ~40–45 mmHg (8).

To assess the role of CYP4A enzymes in modulating vessel responses to reduced PO₂, responses of the arteries to reduced PO₂ were determined before and after incubation of the vessels with 30 μ M of the CYP4A inhibitor dibromododecenyl methylsulfimide (DDMS) for 30 minutes. DDMS (50 mM) stock solution was prepared in anhydrous (200 proof) ethanol that was diluted to the final concentration with PSS. The roles of cyclooxygenase (COX) metabolites and NO in determining vessel responses to reduced PO₂ in control and DDMS-treated vessels were also evaluated by determining the effect of the COX inhibitor indomethacin (1 μ M) and the NO synthase (NOS) inhibitor L-NAME (100 μ M) on the responses of the arteries to reduced PO₂.

Evaluation of Mesenteric Vascular Reactivity to Norepinephrine and Endothelin-1

Additional studies of vascular reactivity were performed in isolated cannulated mesenteric arteries using a tissue culture myograph system (Danish Myo Technology, Aarhus, Denmark). In this system, changes in external diameter of the artery were measured during cumulative increases in norepinephrine (NE; 10⁻⁷–10⁻³ M) or endothelin-1 (ET-1; 10⁻¹⁰–10⁻⁶ M) concentration in the tissue bath. Responses to NE and ET-1 were determined before and after addition of 50 μ M DDMS to the tissue bath to inhibit endogenous production of 20-HETE. An additional series of experiments evaluated vessel responses to NE in DDMS-treated vessels in the presence of 1 μ M WIT003 in the tissue bath. The goal of the latter experiments was to determine whether the responses to NE in DDMS-treated arteries were returned toward control values by addition of a 20-HETE agonist. Time/vehicle control experiments were also performed by repeating NE or ET-1 concentration-response curves in the presence of the DDMS vehicle (0.1% ethanol) in order to verify that any changes in vessel responses in DDMS-treated rats were not due to the DDMS solvent or to nonspecific changes in vessel responses with time.

Cytochrome P450-4A Enzyme Expression

Expression of CYP4A enzyme protein was evaluated using Western blotting techniques. Mesenteric resistance arteries were isolated by microdissection and homogenized in 600 μ L of a solution containing 250 mM sucrose, 1 mM EDTA, and 0.4 μ L protease inhibitor cocktail in a 10 mM potassium phosphate buffer (pH 7.4). Tissue debris and nuclear fragments were removed by centrifugation at 12,000 g for 20 min at 4°C. The amount of dissolved protein in the supernatant was determined with the Bradford assay (Bio-Rad Laboratories, Hercules, CA), using bovine serum albumin as a standard. Proteins from the vessel homogenates were separated by electrophoresis on a 4-15 % SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked for 1 hour in TBS (10 mM Tris, 150 mM NaCl) containing 5 % nonfat dry milk, and then incubated in a TBS solution containing 0.1% Tween-20 (TBST) with a 1:4000 dilution of a goat CYP4A1 antibody (Cat. No. R-PSP151, Nossan Corporation, Yokohama, Japan) that cross reacts with all the CYP4A isoforms. The following day, the membrane was washed with the TBST, and then incubated in a 1:6000 dilution of horseradish peroxidase-coupled donkey anti-goat secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Antigen-antibody reactions were detected using SuperSignal substrate (Pierce, Rockford, IL), exposed to

Kodak Biomax ML film, and developed in a Konica SRX-101 developer. Samples from animals fed LS and HS diet were run on the same gel, to avoid differences arising from gel to gel variation. Densitometry values (pixels) were obtained using UnScanIT 6.1 software (Silk Scientific, Orem, UT). The expression of CYP4A bands for each animal was expressed as % of the pixel density of 17 μ g of β -actin.

20-HETE Production

20-HETE production was measured by LS/MS as described previously (6). In brief, the whole mesenteric bed was isolated and cleaned in PSS-HEPES (5 mM HEPES) buffer (pH 7.4) at 4 °C. The vessels were incubated in 2 ml PSS-HEPES containing 40 μ M arachidonic acid, 1mM NADPH and 40 μ M indomethacin at 37 °C and under 100% O₂ for 90 min. The reactions were terminated by acidification of the reaction buffer to pH 3.5 by 1 M formic acid. Arteries were homogenized in Lysing Matrix Tubes using a FastPrep-24 tissue homogenizer (MP Biomedicals, Solon, OH) with 10 ng internal standard (20-HETE-d6). After the protein concentration of the samples was measured, the organic component of the homogenate was extracted with 3 ml ethyl acetate and dried under N₂. Samples were reconstituted in methanol and resolved by HPLC with mass spectrometry detection. CYP4A activity was expressed as pmol 20-HETE/(90 min $\times$ mg protein).

Statistical Analysis

Vessel responses to NE and ET-1 were expressed as % of control diameter in PSS prior to addition of the vasoconstrictor agonist. Diameter changes in response to reduced PO₂ were calculated as the difference ($\Delta\mu$ m) from NE-pre-constricted control diameter measured during 21% O₂ perfusion/superfusion. The NE or ET-1 concentration that elicited 50% of the maximal constriction (EC₅₀) was calculated from the individual dose responses curves by non-linear regression analysis with a sigmoidal dose response curve fitting method using GraphPad Prism (GraphPAD software Inc., San Diego, USA), and used as an indicator of vessel sensitivity to the agonist.

Data were summarized as mean $\pm$ SEM. Differences between individual groups were evaluated using a Student's t-test for comparison of two groups or ANOVA with a *post hoc* Newman-Keuls test for comparisons of more than two experimental groups. Statistical significance was taken as $P < 0.05$.

RESULTS

Effect of High Salt Diet on Body Weight and Mean Arterial Pressure

Body weights and mean arterial pressures measured under anesthesia for the animals used in this study were similar in rats fed LS vs. HS diet, and are summarized in Table 1.

Effects of High Salt Diet and CYP4A, COX, and NOS Inhibition on Vessel Diameters

There was no significant difference in the resting internal diameter and NE preconstriction level in arteries from SS rats fed LS or HS diet that were used for the reduced PO₂ experiments (Table 1). Inhibition of CYP4A with DDMS ($\pm$ L-NAME or $\pm$ indomethacin), COX inhibition with indomethacin, or NOS inhibition with L-NAME had no effect on the control diameters of arteries isolated from rats fed either HS or LS diet. DDMS pretreatment caused a small but significant decrease in the NE preconstriction level in arteries from HS-fed rats only (Table 1).

Effects of High Salt Diet, CYP4A Inhibition, and WIT003 on Vessel Responses to Norepinephrine

In this study, vessel sensitivity to NE, evaluated as the EC_{50} value, was similar in rats fed LS diet or HS diet (Figure 1 and Table 2). However, inhibition of CYP4A enzymes with DDMS caused a significant reduction in the NE sensitivity of arteries from SS rats fed HS diet, but not those from rats fed LS diet (Figure 1 and Table 2).

In DDMS treated vessels, the 20-HETE agonist WIT003 selectively restored NE sensitivity to pre-treatment values in mesenteric arteries from SS rats fed HS diet, with no effect on vessel sensitivity to NE in SS rats fed LS diet (Figure 1 and Table 2). In the time/vehicle control experiments, application of DDMS solvent (0.1% anhydrous ethanol) did not affect NE concentration-response curves in either of the experimental groups (Figure 1 and Table 2). Maximal responses to NE were unaffected by HS diet, DDMS, WIT003, or DDMS vehicle (Figure 2).

Effects of High Salt Diet and CYP4A Inhibition on Vessel Responses to Endothelin-1

Under control conditions, sensitivity of the vessels to endothelin-1 (ET-1) was more than two orders of magnitude higher than vessel sensitivity to NE in both experimental groups (compare **Figures 1 vs. 2** and **Tables 2 vs. 3**). Elevated dietary salt intake did not cause a significant change in ET-1 sensitivity. In contrast to NE responses; CYP4A inhibition with DDMS did not reduce vessel sensitivity to ET-1, as indicated by the lack of difference in EC_{50} values for ET-1 in the presence and absence of DDMS (Figure 2 and Table 3). In the time/vehicle control experiments, maximal responses to ET-1 decreased by 10-12% nonspecifically (i.e. in the presence of either DDMS or the solvent), with no effect on ET-1 sensitivity (Figure 2 and Table 3).

Effects of High Salt Diet, CYP4A Inhibition, and Inhibition of NO Synthase and Cyclooxygenase on Arterial Responses to Reduced PO₂

Short term HS diet completely eliminated the relaxation of NE-pre-constricted mesenteric arteries in response to reduced PO_2 in SS rats (Figure 3A). In the absence of CYP4A inhibition with DDMS, vessel responses to reduced PO_2 were unaffected by L-NAME, i.e., arteries from rats fed HS diet still failed to relax in response to reduced PO_2 , and the magnitude of vascular relaxation in response to reduced PO_2 was unaffected in arteries from SS rats fed LS diet. By contrast, cyclooxygenase inhibition with indomethacin eliminated vascular relaxation in response to reduced PO_2 in arteries from SS rats fed LS diet, but unmasked a vasodilator response to reduced PO_2 in arteries from SS rats fed HS diet.

Inhibition of 20-HETE production with DDMS restored the vasodilator response to reduced PO_2 in vessels from SS rats fed HS diet, but did not affect the magnitude of vascular relaxation in response to reduced PO_2 in arteries from SS rats fed LS diet (Figure 3B). The vasodilator response to reduced PO_2 that was restored by DDMS in SS rats fed HS diet was eliminated by L-NAME and unaffected by indomethacin, whereas dilation in response to reduced PO_2 in DDMS-treated arteries of SS rats maintained on LS diet was unaffected by L-NAME, but eliminated by indomethacin (Figure 3B).

Effects of High Salt Diet on Expression of CYP4A Enzyme Protein and 20-HETE Production

Expression of CYP4A enzyme protein and 20-HETE production by isolated mesenteric arteries of SS rats fed LS or HS diet are summarized in Table 4. In contrast to our earlier studies of salt-fed Sprague-Dawley rats (48), HS diet caused only a slight (~30%) increase in the expression of the 51 kDa band of CYP4A enzyme protein in arteries from SS rats, which was not significant. HS diet also caused 20-HETE production to increase by ~25% in arteries isolated from SS rats compared to vessels from SS rats fed LS diet, as estimated by

dividing the 20-HETE production per mg of tissue in vessels from rats fed HS diet by the corresponding value in vessels from rats fed LS diet. However, this difference was not significant.

DISCUSSION

Several previous studies have suggested a role for arachidonic acid metabolites of the CYP4A pathway in the development of vascular changes (9,50,52) and elevated blood pressure (23,41,52) in hypertension. The present study reports several new findings regarding the role of CYP4A metabolites in contributing to altered vascular regulation during the very early stages elevated dietary salt intake in the Dahl salt-sensitive rat, a widely used rodent model of salt-sensitive hypertension in humans. First, inhibition of 20-HETE production with DDMS selectively reduces vascular NE sensitivity and restores the dilation in response to reduced PO₂ that is lost in mesenteric arteries from SS rats fed HS diet. Short term HS diet tended to increase 20-HETE production in arteries of SS rats (by ~25%), although this difference was not significant and, in contrast to earlier studies of Sprague-Dawley rats (48), did not cause a significant increase in the expression of CYP4A enzymes in mesenteric resistance arteries of SS rats. Finally, during CYP4A inhibition, exogenous addition of the 20-HETE agonist WIT003 prevented the reduction in NE sensitivity in mesenteric resistance arteries from SS rats fed HS diet, with no significant effect on NE sensitivity in arteries from SS rats fed LS diet. The latter findings indicate that 20-HETE modulates the responses of mesenteric resistance arteries to NE and reduced PO₂ in HS-fed SS rats, and that vessel sensitivity to 20-HETE and/or 20-HETE production is increased in the very early stages of elevated dietary salt intake in SS rats.

Norepinephrine- and Endothelin-1-Induced Constriction

A variety of studies in the literature indicate that vessel sensitivity to adrenergic agonists (2,18,38,58) and other vasoconstrictors, e.g. angiotensin II (9) and vasopressin (44), but not ET-1 (44), is increased in various hypertensive models. In SS rat studies where blood pressure (2), isometric contractile force in aortas (38) or perfusion pressure of mesenteric vascular beds (18) are studied, elevated dietary salt intake results in an enhanced responsiveness to NE. In contrast to those reports, the respective vasoconstrictor sensitivities to NE and ET-1 in the present study were similar in small mesenteric arteries from SS rats fed LS and HS diet. Despite the similar EC₅₀ values for NE and ET-1 in vessels from SS rats fed LS vs. HS diet, CYP4A inhibition by DDMS caused a significant and selective reduction in NE sensitivity in SS rats fed HS diet only. The latter finding indicates that CYP4A products in the vessel wall modulate NE but not ET-1 induced vasoconstriction in mesenteric resistance arteries from SS rats fed HS diet, which is similar to previous reports (1) showing that CYP4A metabolites modulate phenylephrine- (but not ET-1-) induced constriction of mesenteric resistance arteries from aged rats compared to young controls.

The failure of HS diet to influence ET-1 sensitivity in arteries of salt-fed SS rats in the present study is surprising in light of the findings of Oyekan et al. (39) showing that ET-1-induced increases in 20-HETE production play a major role in mediating cardiovascular and renal injury in rats with DOCA-salt hypertension. The reasons for the failure of CYP4A inhibition to reduce ET-1 sensitivity in arteries of salt fed SS rats in our experiments are unclear, but may include any or all of the following: 1) differences in the relative contribution of CYP4A metabolites to vascular changes in different forms of hypertension (19,21); 2) the emergence of alternative mechanisms to mediate vasoconstrictor responses to ET-1 in addition to CYP4A (19,21,34), 3) CYP4A isoform-dependent differences in 20-HETE production depending on the location or regulation of specific CYP4A isoforms (15,33,36); 4) the participation of second messengers other than 20-HETE in mediating ET-1-induced contractions of the vessels (1,24,57); or 5) differences in the

electrophysiological responses to NE and ET-1 acting in the face of a partially depolarized vascular smooth muscle (VSM) transmembrane potential (E_m). In the latter scenario, the ability of 20-HETE to depolarize VSM (E_m) by inhibiting Ca^{2+} -activated K^+ channels in the cell membrane (22) would sensitize the VSM by moving E_m closer to the threshold for opening voltage-activated Ca^{2+} channels. Therefore, the larger depolarization produced by NE (13) compared to that produced by ET-1 (7) would be more likely to reach the threshold to activate contraction in arterial smooth muscle where resting E_m is more depolarized by higher levels of 20-HETE (and/or increased 20-HETE sensitivity) during elevated dietary salt intake.

CYP4A Expression, 20-HETE Production, and 20-HETE Sensitivity in Arteries of SS Rats Fed HS vs. LS Diet

In the present study, short term HS diet caused only a slight (~30%) increase in CYP4A protein in arteries of SS rats, which was not significant. These findings are in contrast to previous studies by our laboratory (48) showing that HS diet causes a significant increase in the expression of 51 kDa band of CYP4A enzyme protein in mesenteric resistance arteries of Sprague-Dawley rats. 20-HETE production also tended to be higher (by ~25%) in arteries from SS rats fed HS diet, compared to LS controls (Table 4), although this difference was not significant. Taken together, these findings suggest that the intrinsic ability of the arteries to produce 20-HETE via the CYP4A system is not enhanced by HS diet in SS rats. However, because these measurements of 20-HETE production were made under optimum conditions for 20-HETE formation, they do not eliminate the possibility that HS diet can enhance 20-HETE production *in vivo* via other mechanisms, e.g., substrate availability, in the SS rats. Therefore, a contribution of enhanced 20-HETE production to the DDMS-sensitive alterations in vascular responses to NE and reduced PO_2 in the present studies cannot be excluded.

In these experiments, exogenous addition of the 20-HETE agonist WIT003 to the tissue bath in the presence of DDMS to inhibit endogenous 20-HETE production selectively increased NE sensitivity in arteries from salt-fed SS rats. The latter observation suggests that 20-HETE plays a greater role in modulating vasoconstrictor responses to NE in SS rats fed short term HS diet compared to LS controls. These findings also indicate that vessel sensitivity to 20-HETE is increased in arteries of SS rats fed short term HS diet compared to SS rats fed LS diet. In conjunction with the tendency for 20-HETE production to be increased in mesenteric arteries of SS rats fed HS diet (Table 4), these findings provide further evidence that CYP4A enzymes play a greater role in modulating vessel responses to some vasoconstrictor stimuli in salt-sensitive hypertension. Consistent with this interpretation, the constriction of cremasteric arterioles in response to angiotensin II and/or elevated PO_2 is reduced to a greater extent by CYP4A inhibition both in RRM salt-loading hypertension (9) and in SS rats fed HS diet (49).

Response to Reduced PO_2

In addition to the effects of CYP4A metabolites in modulating vasoconstrictor responses to NE in mesenteric arteries of SS rats, inhibition of CYP4A enzymes had a dramatic effect on vessel responses to reduced PO_2 . In these experiments, short term HS diet completely eliminated the dilation of mesenteric arteries in response to reduced PO_2 in SS rats. CYP4A inhibition with DDMS restored vasodilation in response to reduced PO_2 in vessels from SS rats fed HS diet, but had no effect on vessel responses to reduced PO_2 in SS rats fed LS diet (Figure 3B). These observations suggest that changes in 20-HETE production and/or increased 20-HETE sensitivity also contribute to the loss of vascular relaxation in response to reduced PO_2 in mesenteric resistance arteries of SS rats fed short-term HS diet.

A novel and surprising finding in the present study was that the vasodilator response to reduced PO_2 that was unmasked by CYP4A enzyme inhibition in SS rats fed HS diet is mediated by an entirely different mechanism, specifically NO, rather than vasodilator metabolites of the cyclooxygenase (COX) pathway of arachidonic acid metabolism that mediate vascular relaxation in response to reduced PO_2 in arteries of SS rats fed LS diet (Figure 3B). Although previous studies have shown that COX metabolites, NO, and changes in 20-HETE levels can all play a role in mediating vascular relaxation in response to reduced PO_2 in skeletal muscle resistance arteries (10), COX metabolites are normally the primary mediators of the vasodilator response to reduced PO_2 in rat arterioles and resistance arteries (8, 31, 35) with little contribution from NO (10). However, previous studies indicate that CYP4A metabolites play a larger role in modulating the response of skeletal muscle resistance arteries to reduced PO_2 in SS rats fed HS diet for 4 weeks compared to vessels from SS rats maintained on LS diet (11). The observation that CYP4A inhibition unmasks a NO-mediated dilation in response to reduced PO_2 in SS rats fed short term HS diet is similar to the finding of Kerkhof et al. (17), who reported that CYP4A inhibition revealed a NO-mediated dilation in response to reduced PO_2 in isolated first order cremasteric arterioles from normotensive Wistar rats. The precise mechanism(s) by which CYP4A enzymes and 20-HETE counteract vasodilation in response to reduced PO_2 and the reason for the change from COX-dependent to NO-mediated relaxation in response to reduced PO_2 in DDMS-treated arteries from SS rats fed HS diet have yet to be determined.

In untreated arteries of SS rats fed HS diet, NO inhibition with L-NAME has no effect on vessel responses to reduced PO_2 (no dilation before or after L-NAME treatment), while inhibition of cyclooxygenase (COX) with indomethacin unmasks a vasodilator response to reduced PO_2 in the absence of CYP4A inhibition with DDMS (Figure 3A). The latter observation is similar to the effect of HS diet on the response of middle cerebral arteries to reduced PO_2 in Sprague-Dawley rats, where thromboxane and possibly other vasoconstrictor metabolites of the COX pathway contribute to the loss of vascular relaxation in response to reduced PO_2 (32). However, inhibition of CYP4A enzymes either eliminates the formation of vasoconstrictor COX metabolites in arteries of SS rats fed HS diet, and/or increases nitric oxide to levels that can overcome the vasoconstrictor effects of COX metabolites during exposure to reduced PO_2 . Relevant to this hypothesis, CYP4A enzymes have been identified as a potential source of reactive oxygen species (ROS) (56); and recent studies (50) have shown that over-expression of the CYP4A2 isoform in renal interlobar arteries leads to endothelial dysfunction and increased oxidative stress in Sprague-Dawley rats. There is also evidence that CYP4A enzymes and 20-HETE contribute to NOS uncoupling (3), which would lead to increased superoxide production and a concomitant reduction of NO levels, as previously demonstrated in aortas of Sprague-Dawley rats fed HS diet (60). Under these conditions, inhibiting 20-HETE production with DDMS would augment the NO-dependent component of vasodilation in response to reduced PO_2 in arteries from rats fed a HS diet (10).

Summary and Conclusions

The present study shows that inhibition of CYP4A enzymes selectively reduces NE sensitivity in arteries of SS rats fed short term HS diet and restores the vascular relaxation in response to reduced PO_2 that is lost in SS rats fed HS diet, with no effect on the magnitude of dilation in response to reduced PO_2 in SS rats fed LS diet. Inhibition of CYP4A enzymes also unmasked a NO-dependent mechanism of vascular relaxation in response to reduced PO_2 in SS rats fed HS diet, rather than the cyclooxygenase-dependent mechanism that mediates dilation of the arteries in response to reduced PO_2 (insensitive to CYP4A inhibition) in SS rats fed LS diet. In contrast to earlier studies in Sprague-Dawley rats (48), short term HS diet did not appear to cause a significant change in the expression of CYP4A

enzyme protein in arteries from SS rats. While HS diet appeared to cause a modest increase in 20-HETE production under optimum conditions for 20-HETE synthesis, this difference was not significant. However, exogenous addition of the 20-HETE agonist WIT003 selectively restored vasoconstrictor sensitivity to NE in arteries of SS rats fed HS diet, suggesting that vessel sensitivity to 20-HETE may be increased in mesenteric resistance arteries of SS rats fed short-term HS diet. Taken together, these findings indicate that the 20-HETE/CYP4A system contributes to alterations in the sensitivity of mesenteric resistance arteries to reduced PO₂ and NE during the earliest stages of elevated dietary salt intake in the SS rat, which is already predisposed to endothelial dysfunction in the absence of HS diet or an elevation in arterial blood pressure (5). Eventually, these functional changes in the vessel wall could exacerbate the development of vascular dysfunction and an elevated peripheral vascular resistance in SS rats, and accelerate the development of the elevated blood pressure in this genetic model of human salt-sensitive hypertension.

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Figure 1.

Norepinephrine concentration-response curves for mesenteric resistance arteries from Dahl SS rats fed low salt (LS, n=7; left) or high salt (HS, n=7; right) diet as a % of control diameter (CTRL; 100%). Norepinephrine-induced contractions were measured either in physiological salt solution (Control); in the presence of DDMS solvent only; in the presence of the CYP4A inhibitor DDMS; or in the presence of DDMS plus the 20-HETE agonist WIT003. Mean $-\log EC_{50} \pm SEM$ values are summarized in Table 2.

Figure 2.

Endothelin-1 concentration-response curves for mesenteric resistance arteries from Dahl SS rats fed low salt (LS, n=7; left) or high salt (HS, n=7; right) diet. Data are expressed as % of control diameter (CTRL; 100%). Endothelin-1 induced contractions were measured in physiological salt solution (Control); in the presence of DDMS solvent only; or in the presence of the CYP4A inhibitor DDMS. Mean $-\log EC_{50} \pm SEM$ values are summarized in Table 3.

Figure 3.

Effect of high salt diet on the response to reduced PO₂ in norepinephrine pre-constricted mesenteric resistance arteries from Dahl SS rats fed low salt (LS, n=5-12) or high salt (HS, n=7-12) diet. In the absence of CYP4A inhibition with DDMS (**A**), HS diet completely eliminated vasodilator responses to reduced PO₂ compared to LS controls. In untreated arteries (no DDMS), cyclooxygenase inhibition with indomethacin unmasked a vasodilator response to reduced PO₂ in arteries from SS rats fed HS diet, and eliminated vascular relaxation in response to reduced PO₂ in arteries from SS rats fed LS diet. Selective inhibition of CYP4A with DDMS (**B**) restored vasodilation in response to reduced PO₂ in arteries from SS rats fed HS diet without affecting the response to reduced PO₂ in arteries of SS rats fed LS diet. Vasodilation in response to reduced PO₂ was prevented by cyclooxygenase inhibition with indomethacin and NO synthase inhibition with L-NAME in DDMS-treated arteries from SS rats fed LS and HS diet, respectively. Data are plotted as mean increase (μm) ± SEM from NE pre-contracted diameter. * and † significantly different (P < 0.05) from LS and HS control, respectively.

Table 1

Summary of body weights and mean arterial pressures measured under anesthesia for SS rats fed low salt or high salt diet used in this study. Internal diameters (μm) and NE precontraction levels (%) of mesenteric arteries used for reduced O_2 experiments with various inhibitor or agonist treatments are also included

Group	Low Salt	High Salt
Body weight (g)	297.4 $\pm$ 4.5 (n=56)	306.9 $\pm$ 4.5 (n=58)
Blood pressure (mmHg)	121.2 $\pm$ 2.1 (n=44)	126.2 $\pm$ 2.8 (n=44)
Internal diameter (μm)/NE precontraction level (%)		
Combined Control	248.4 $\pm$ 5.1 (n=29)/ 49.3$\pm$1.2 (n=23)	240.4 $\pm$ 4.7 (n=30)/ 52.5$\pm$1.2 (n=23)
Control	236.7 $\pm$ 6.7 (n=12)	239.3 $\pm$ 7.1 (n=12)
+Indo	236.8 $\pm$ 6.8/ 49.2$\pm$1.7	239.0 $\pm$ 7.2/ 47.8$\pm$1.2
Control	239.7 $\pm$ 6.8 (n=12)	236.7 $\pm$ 8.0 (n=12)
+L-NAME	240.6 $\pm$ 7.0/ 47.3$\pm$1.6	237.9 $\pm$ 8.4/ 50.9$\pm$3.3
Control	250.2 $\pm$ 7.3 (n=17)	240.1 $\pm$ 6.5 (n=18)
+DDMS	249.1 $\pm$ 7.4/ 48.7$\pm$1.4	240.1 $\pm$ 6.3/ 53.1$\pm$1.6 *
Control	234.0 $\pm$ 12.7 (n=6)	222.9 $\pm$ 8.2 (n=7)
+DDMS	234.3 $\pm$ 13.6	224.0 $\pm$ 8.0
+DDMS+Indo	234.5 $\pm$ 13.8/ 48.4$\pm$1.7	223.6 $\pm$ 7.9/ 46.7$\pm$1.6
Control	226.0 $\pm$ 7.3 (n=5)	225.1 $\pm$ 9.0 (n=7)
+DDMS	226.4 $\pm$ 7.3	225.7 $\pm$ 9.5
+DDMS+L-NAME	228.2 $\pm$ 6.8/ 45.5$\pm$2.9	226.0 $\pm$ 9.4/ 47.9$\pm$2.2

Data are summarized as mean $\pm$ SEM.

* Significantly different ($P < 0.05$) from LS.

Table 2

–logEC₅₀ values for norepinephrine-induced contractions of mesenteric arteries from SS rats fed low salt (LS) or high salt (HS) diet

Group	LS	HS
Control	5.30 ± 0.09	5.36 ± 0.09
DDMS solvent	5.46 ± 0.06	5.34 ± 0.10
DDMS	5.12 ± 0.11	4.96 ± 0.12*
DDMS+WIT003	5.10 ± 0.14	5.42 ± 0.08

Mean –logEC₅₀ ± SEM values calculated for the corresponding norepinephrine concentration-response curves shown in Figure 1 for mesenteric resistance arteries from Dahl SS rats fed low salt (LS) and high salt (HS) diet (n=7 for each group).

* Significantly different (P<0.05) from HS Control.

Table 3

–logEC₅₀ values for endothelin-1-induced contractions of mesenteric arteries from SS rats fed low salt (LS) or high salt (HS) diet

Group	LS	HS
Control	7.64 ± 0.21	7.64 ± 0.15
DDMS	7.90 ± 0.21	7.73 ± 0.16
DDMS solvent	7.88 ± 0.33	7.89 ± 0.30

Mean –logEC₅₀ ± SEM values calculated for the corresponding endothelin-1 concentration-response curves shown in Figure 2 for mesenteric resistance arteries from Dahl SS rats fed low salt (LS) and high salt (HS) diet (n=7 for each group).

Table 4

Expression of CYP4A enzyme protein and 20-HETE production in mesenteric arteries of Dahl SS rats fed low salt (LS) or high salt (HS) diet

Group	LS	HS
CYP4A expression (% β actin)	99.5 $\pm$ 19.3 (n=8)	130.5 $\pm$ 19.4 (n=8)
20-HETE production (pmol min ⁻¹ mg protein ⁻¹)	0.04 $\pm$ 0.003 (n=11)	0.05 $\pm$ 0.005 (n=12)

Data are given as mean $\pm$ SEM.