High dietary salt reduces the contribution of 20-HETE to arteriolar oxygen responsiveness in skeletal muscle

Paul J. Marvar,1 John R. Falck,2 and Matthew A. Boegehold1

1Department of Physiology and Pharmacology and Center for Interdisciplinary Research in Cardiovascular Sciences, West Virginia University School of Medicine, Morgantown, West Virginia; and 2Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas

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Marvar PJ, Falck JR, Boegehold MA. High dietary salt reduces the contribution of 20-HETE to arteriolar oxygen responsiveness in skeletal muscle. Am J Physiol Heart Circ Physiol 292: H1507–H1515, 2007.—The coupling of tissue blood flow to cellular metabolic demand involves oxygen-dependent adjustments in arteriolar tone, and arteriolar responses to oxygen can be mediated, in part, by changes in production of 20-HETE. In this study, we examined the long-term effect of dietary salt on arteriolar oxygen responsiveness in the exteriorized, superfused rat spinotrapezius muscle and the role of 20-HETE in this responsiveness. Rats were fed either a normal-salt (NS, 0.45%) or high-salt (HS, 4%) diet for 4–5 wk. There was no difference in steady-state tissue PO2 between NS and HS rats, and elevation of superflusate oxygen content from 0% to 10% caused tissue PO2 to increase by the same amount in both groups. However, the resulting reductions in arteriolar diameter and blood flow were less in HS rats than NS rats. Inhibition of 20-HETE formation with N-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS) or 17-octadecynoic acid (17-ODYA) attenuated oxygen-induced constriction in NS rats but not HS rats. Exogenous 20-HETE elicited arteriolar constriction that was greatly reduced by the large-conductance Ca2+-activated potassium (KCa) channel inhibitors tetraethylammonium chloride (TEA) and iberiotoxin (IbTx) in NS rats and a smaller constriction that was less sensitive to TEA or IbTx in HS rats. Arteriolar responses to exogenous angiotensin II were similar in both groups but more sensitive to inhibition with DDMS in NS rats. Norepinephrine-induced arteriolar constriction was similar and insensitive to DDMS in both groups. We conclude that 20-HETE contributes to oxygen-induced constriction of skeletal muscle arterioles via inhibition of KCa channels and that a high-salt diet impairs arteriolar responses to increased oxygen availability due to a reduction in vascular smooth muscle responsiveness to 20-HETE.

20-hydroxyeicosatetraenoic acid; vascular control; blood flow; NaCl

LOCAL OXYGEN AVAILABILITY is an important determinant of microvascular resistance and tissue blood flow (10, 17, 22, 41), and consumption of a high-salt diet can lead to changes in resistance vessel function that alter the relationship between oxygen and vascular tone. For example, small gracilis muscle feed arteries from rats fed high salt for as little as 3 days consistently show an impaired dilator response to reduced oxygen levels (47, 48). However, the effect of oxygen on the tone of smaller arterioles in rat cremaster muscle is unchanged after 3 days on a high-salt diet (13). This suggests that either a longer period of high-salt intake is required to alter the oxygen responsiveness of these more distal resistance vessels or that there is a fundamental difference between extraparenchymal arteries and intramuscular arterioles in the susceptibility of oxygen-sensitive tone to dietary salt. The first aim of this study was to distinguish between these possibilities by determining whether a prolonged period of high-salt intake can change the relationship between oxygen and vascular tone in the arteriolar network of skeletal muscle.

The mechanisms through which oxygen can influence resistance vessel tone have long been of interest to those studying local blood flow regulation (10, 22). Mounting evidence suggests that the enzyme cytochrome P-450 4A (CYP450 4A) ω-hydroxylase, which is present in skeletal muscle arterioles as well as in the surrounding muscle fibers (25), plays a pivotal role in arteriolar oxygen responsiveness. Immediately following an elevation or reduction in local PO2, this enzyme can respectively increase or decrease production of the vasoconstrictor 20-HETE (19, 34), and an explicit contribution of 20-HETE to oxygen-induced constriction has been documented in hamster retractor muscle arterioles (34), rat and hamster cremaster muscle arterioles (12, 13, 19, 25, 34), and isolated rat gracilis muscle feed arteries (15). There is also a role for 20-HETE in the oxygen-dependent control of arteriolar tone in non-muscle vascular beds (19, 50). More recent findings indicate that the expression of CYP450 4A protein and 20-HETE production are both increased in mesenteric resistance arteries of rats fed a high-salt diet and that these vessels also exhibit an impaired responsiveness to changes in oxygen availability (46). However, given the functional differences that can exist among resistance vessels in different vascular beds (21, 34), these results cannot be automatically extrapolated to skeletal muscle. Therefore, a second aim of this study was to assess whether high-salt intake alters the specific contribution of the CYP450 4A/20-HETE pathway to oxygen-dependent changes in the tone of skeletal muscle arterioles.

METHODS

At 3 wk of age, male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were placed on a whole-grain diet containing either 0.45% NaCl (normal salt, NS) or 4% NaCl (high salt, HS) by weight (NS diet, TD88311; HS diet, TD92034; Teklad, Madison, WI). All rats were studied after 4–5 wk on their respective diets and were therefore 7–8 wk old at this time. All surgical and experimental procedures were approved by the West Virginia University Animal Care and Use Committee.

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Surgical Preparation and Intravital Microscopy

Each rat was anesthetized with thiopental sodium (100 mg/kg ip) and placed on a heating pad to maintain a 37°C rectal temperature. The trachea was intubated to ensure a patent airway, and the right carotid artery was cannulated to measure arterial pressure. The right spinotrapezius muscle was exteriorized for microscopic observation as previously described (6), leaving its innervation and all feed vessels completely intact. Throughout the surgery and subsequent experimental period, the muscle was continuously superfused with an electrolyte solution (119 mM NaCl, 25 mM NaHCO₃, 6 mM KCl, and 3.6 mM CaCl₂) warmed to 35°C and equilibrated with 95% N₂:5% CO₂ (pH 7.35–7.40). Superfusate flow rate was maintained at 4–6 ml/min to minimize equilibration with atmospheric oxygen (7).

The animal preparation was transferred to the stage of an Olympus BX50WI intravital microscope (Hyde Park, NY) fitted with a charge-coupled device video camera (Dage-MTI, Michigan City, IN). Video images were displayed on a Sony high-resolution video monitor and videotaped for off-line analysis. Observations were made with an Olympus ×20 water immersion objective (final video image magnification, ×1,460). Arteriolar center line red blood cell velocities were measured online with an optical Doppler velocimeter (Cardiovascular Research Institute, Texas A&M University, Temple, TX), and arteriolar inner diameters were measured during videotape replay with a video caliper (Cardiovascular Research Institute).

Inhibition of CYP450 4A ω-Hydroxylase

To evaluate the role of CYP450 4A enzymes in regulating arteriolar tone, we used two different inhibitors: 17-octadecynoic acid (17-ODYA, Sigma Chemical, St. Louis, MO), a suicide substrate inhibitor of CYP450 4A ω-hydroxylases (19, 51) that can also block CYP450 epoxygenase activity (51), and the more specific inhibitor of CYP450 4A ω-hydroxylase, N-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS) (2). In the first set of experiments, 17-ODYA (1 × 10⁻⁵ M) was topically applied to the muscle for 30 min while superfusion was stopped. In other experiments, a 5 × 10⁻⁵ M solution of DDMS was topically applied to the muscle (with superfusion stopped) for 30 min, followed by continuous superfusion of the muscle with 1 × 10⁻⁵ M DDMS for the rest of the experimental period. These inhibitors have been shown to be effective in other in vivo studies examining the role of 20-HETE in skeletal muscle microvascular control (33).

Application of Exogenous Vasocostricators

To determine whether the sensitivity of spinotrapezius muscle arterioles to 20-HETE is different between dietary groups, increasing concentrations of 20-HETE (1 × 10⁻⁹ M to 5 × 10⁻⁸ M, Sigma) were added directly to the muscle superfusate. Before 20-HETE application, the muscle was superfused with 1 × 10⁻⁵ M 17-ODYA, to inhibit endogenous 20-HETE formation, and the lipooxygenase inhibitor baicalin (1 × 10⁻⁵ M, Sigma) plus the cyclooxygenase inhibitor indomethacin (1 × 10⁻⁶ M, Sigma), to prevent the metabolism of exogenous 20-HETE to other products (33).

Previous studies suggest that ANG II and norepinephrine (NE) may elicit vasocostriction, in part, through 20-HETE production (1, 20, 24). Therefore, we also assessed arteriolar responsiveness to ANG II (1 × 10⁻⁹ M and 1 × 10⁻⁸ M, Sigma) and NE (1 × 10⁻⁷ M and 1 × 10⁻⁸ M, Sigma) before and after inhibition of 20-HETE formation with DDMS.

Inhibition of Ca²⁺-Activated Potassium Channels

20-HETE causes vasocostriction, in part, by inhibiting the activity of large-conductance Ca²⁺-activated potassium (KCa) channels (52). Therefore, we also assessed the effect of iberiotoxin (IbTx, Sigma), at a superfusate concentration of 1 × 10⁻⁶ M, or tetraethylammonium chloride (TEA, Sigma), at a superfusate concentration of 1 × 10⁻⁵ M, on arteriolar responses to exogenous 20-HETE. At this concentration, TEA has been shown to selectively block KCa channels, without effects on other potassium channels (37). IbTx was also used to assess the role of KCa channels in arteriolar responses to oxygen (see below).

Western Analysis for CYP450 4A ω-Hydroxylase

Western blots were performed to measure the expression of CYP450 4A ω-hydroxylase in isolated microvessels and in whole spinotrapezius muscle of rats from each dietary group. After induction of thiopental sodium anesthesia, the right and left spinotrapezius muscles were removed, rinsed in 4°C electrolyte solution, and immediately frozen in liquid nitrogen. In other rats, the right and left spinotrapezius muscles were removed, rinsed in the electrolyte solution, and pinned out in a Silastic-coated petri dish filled with 100% methanol at 4°C. Arcade arterioles (see below) were then excised from the surrounding parenchyma and immediately frozen in liquid nitrogen. Whole skeletal muscles or isolated arterioles were homogenized on ice with T-PER buffer (Pierce, Rockford, IL) and then centrifuged at 10,000 rpm for 5 min at 4°C, with the supernatant stored at −80°C until use. One microgram of vessel protein (measured with a Bio-Rad protein assay kit) from each dietary group was separated on a NuPage 4–12% bis-Tris polyacrylamide gel (Invitrogen, San Diego, CA) and transferred to a nitrocellulose membrane. The membrane was probed with rabbit polyclonal antibody for CYP450 4A (1:1,500; Affinity Bioreagents, Golden, CO) and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3,000; Santa Cruz Biotechnology, Santa Cruz, CA). After treatment with SuperSignal (Pierce), membranes were exposed to autoradiography film. X-ray images were digitally captured, and optical densitometry was performed. To ensure equal protein loading, GAPDH was measured as a control protein.

Experimental Protocols

Protocol 1: arteriolar responses to increased oxygen availability

In rat spinotrapezius muscle, there is a network of interconnected arcade arterioles that extends throughout the muscle (28). After exteriorization of the spinotrapezius muscle and a 30-min postsurgical equilibration period, one to three arcade arterioles were chosen for study, and baseline measurements of resting arteriolar diameter and flow were determined. Then, to assess oxygen responsiveness, the superfusate was switched from the 0% oxygen solution (equilibrated with 95% N₂:5% CO₂) to a 10% oxygen solution (equilibrated with 85% N₂:5% CO₂), and after a 10-min equilibration period, arteriolar diameters and blood flows were remeasured.

Under each superfusate, muscle bath and tissue PO₂ were measured using an Apollo 4000 free radical analyzer (World Precision Instruments, Sarasota, FL) with carbon fiber oxygen mini-sensors (100-μm tip diameter) that were calibrated immediately before and after each experiment. For calibration, sensor currents were monitored in superfusion solution (see above) equilibrated with 0%, 5%, and 10% oxygen at 35°C. Measurements from sensors whose pre- and postexperimental calibration values differed by more than 5% were discarded from the final data set. Tissue PO₂ was measured near the venous end of capillary networks.

Protocol 2: role of CYP450 4A ω-hydroxylase or KCa channels in arteriolar responses to oxygen

To assess the role of 20-HETE in oxygen-induced arteriolar constriction, we measured arteriolar diameter and flow responses to the 10% oxygen superfusate, as described above, before and during exposure to DDMS or 17-ODYA. In a second series of experiments, the contribution of KCa channels to oxygen-induced arteriolar constriction was assessed by measuring diameter responses to 10% oxygen before and during exposure to IbTx. The contribution of KCa channels to the effects of 20-HETE on arteriolar diameter was assessed in a third series of experiments. In these experiments, arteriolar diameters were measured at rest and after exogenous 20-HETE application, and these
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measurements were then repeated following addition of TEA or IbTx to the superfusate. To assess the role of 20-HETE in the vasoconstrictor effects of ANG II and NE, arteriolar diameters were measured before and after either ANG II (1 × 10^{-9}–1 × 10^{-8} M) or NE (1 × 10^{-6}–1 × 10^{-5} M) was added to the bath. Following a washout period to allow for restoration of normal arteriolar tone, responses to the same concentrations of ANG II and NE were reexamined in the presence of DDMS.

At the end of most in vivo experiments, 1 × 10^{-4} M adenosine was added to the superfusate to abolish microvascular tone for measurement of passive arteriolar diameter.

Data and Statistical Analysis

Arteriolar diameter (D; in μm) and center line red blood cell velocity (V_{cl}; in mm/s) were measured. Mean red blood cell velocity (V_{mean}) was calculated as V_{cl}/1.6, where 1.6 is the ratio of center line red blood cell velocity to mean velocity for vessels >10 μm in diameter (53). Paired values of D and V_{mean} were used to calculate arteriolar volume flow (Q; in nl/s), where Q = V_{mean}[\pi(D^2/4)].

All data are reported as means ± SE. Statistical analysis was performed by commercially available software (SigmaStat, SPSS, Chicago, IL). Two-way, repeated-measures ANOVA was used to determine the effects of diet, treatment, and diet-treatment interactions on measured variables, and Student’s t-test was used for statistical comparisons between groups. For all ANOVA procedures, the Student’s t-test was used for statistical comparisons between groups. Significance was assessed at the 95% confidence level (P < 0.05) for all tests.

RESULTS

At the time of study, rats fed the high-salt diet had an average body weight of 293 ± 4 g and an average mean arterial pressure of 124 ± 3 mmHg (n = 40). These values were not significantly different from those of rats fed the normal diet (294 ± 4 g and 121 ± 3 mmHg, respectively; n = 40). Table 1 displays the characteristics of all arterioles studied by in vivo microscopy in protocols 1 and 2. Under control conditions (0% oxygen superfusate), there were no significant differences between dietary groups in steady-state arteriolar diameter, volume flow or calculated vascular tone, or in passive arteriolar diameter measured after abolition of tone with adenosine. As shown in Table 2, neither 17-ODYA nor DDMS had a significant effect on steady-state arteriolar diameters or flows in either group.

With the 0% oxygen superfusate, bath PO2 immediately above the muscle surface averaged 14 ± 1 mmHg, with tissue PO2 averaging 26.9 ± 2.0 mmHg in NS rats and 29.1 ± 3.5 mmHg in HS rats. These tissue values were not significantly different. Changing to the 10% oxygen superfusate increased bath PO2 to 68 ± 3 mmHg, and under these conditions, tissue PO2 significantly increased to 33.4 ± 2.6 mmHg in NS rats and 36.2 ± 2.0 mmHg in HS rats. These values were also not significantly different between groups. Arteriolar responses to these increases in oxygen availability are shown in Fig. 1. Arteriolar constriction and a reduction in blood flow were observed in both dietary groups, but when compared with the NS rats, these responses were significantly attenuated in the HS rats. The effects of inhibiting 20-HETE formation on arteriolar

Table 1. Arteriolar characteristics at time of study

<table>
<thead>
<tr>
<th>Dietary Group</th>
<th>Resting Diameter, μm</th>
<th>Passive Diameter, μm</th>
<th>Resting Vascular Tone, % of Maximum</th>
<th>Resting Volume Flow, nl/s</th>
</tr>
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<tbody>
<tr>
<td>Normal salt</td>
<td>20.4 ± 0.5</td>
<td>60.1 ± 2.5</td>
<td>64.1 ± 1.6</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>High salt</td>
<td>20.0 ± 0.4</td>
<td>54.4 ± 2.1</td>
<td>60.9 ± 1.9</td>
<td>1.9 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. For normal salt group, n = 106 vessels for resting diameter, 75 vessels for passive diameter and vascular tone, and 65 vessels for resting volume flow. For high-salt group, n = 121 vessels for resting diameter, 82 vessels for passive diameter and vascular tone, and 59 vessels for resting volume flow.

Table 2. Effect of ODYA and DDMS on resting arteriolar diameter and volume flow

<table>
<thead>
<tr>
<th></th>
<th>Diameter, μm</th>
<th>Flow, nl/s</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal Salt</td>
<td>High Salt</td>
</tr>
<tr>
<td>Control</td>
<td>22.6 ± 1.4</td>
<td>18.6 ± 1.0</td>
</tr>
<tr>
<td>ODYA</td>
<td>22.2 ± 1.4</td>
<td>20.6 ± 1.5</td>
</tr>
<tr>
<td>Control</td>
<td>17.9 ± 0.2</td>
<td>16.0 ± 0.3</td>
</tr>
<tr>
<td>DDMS</td>
<td>17.5 ± 0.8</td>
<td>16.9 ± 1.0</td>
</tr>
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Values are means ± SE. For 17-octadecynoic acid (ODYA; 1 × 10^{-5} M) effect, n = 16 vessels for normal-salt group and 9 vessels for high-salt group. For N-methylsulfonyl-12,12-dibromododec-11-ename (DDMS; 5 × 10^{-5} M) effect, n = 9 vessels for normal salt group and 7 vessels for high-salt group.
responses to increased oxygen are shown in Fig. 2. Despite having no effect on resting arteriolar tone, both 17-ODYA and DDMS markedly decreased the oxygen-induced constriction and flow reduction in NS rats (Fig. 2, left). In contrast, neither of these inhibitors had a significant effect on arteriolar responses to increased superfusate oxygen in HS rats (Fig. 2, right).

A representative CYP450 4A ω-hydroxylase Western blot from spinotrapezius muscle protein is shown in Fig. 3. Protein expression was detected at the 51-kDa band, which corresponds to the CYP450 4A protein. Densitometric analysis revealed no difference between dietary groups in the expression of CYP450 4A protein in whole muscle (Fig. 3). Western blotting of protein from the arcade arterioles in this muscle revealed that CYP450 4A protein is expressed in the arteriolar walls of rats fed both diets (data not shown). However, because these vessels, even when pooled, yielded only a very small amount of protein, we were not able to obtain enough blots for a statistically relevant densitometric analysis of any diet-related differences in protein expression.

Figure 4 illustrates arteriolar diameter changes in response to various concentrations of exogenously applied 20-HETE. Exogenous 20-HETE elicited arteriolar constriction in both groups, but the magnitude of these responses was significantly less in rats fed high salt.

Under control conditions, resting arteriolar diameters were significantly reduced by the KCa channel inhibitors TEA and IbTx in both dietary groups (NS, preinhibitor: 21.0 ± 0.5 μm, postinhibitor: 15.6 ± 1.4 μm; HS, preinhibitor: 20.5 ± 0.5 μm, postinhibitor: 14.7 ± 1.2 μm). The effects of TEA and IbTx on arteriolar responses to exogenous 20-HETE are shown in Fig. 5. Both of these inhibitors greatly reduced the responses to $1 \times 10^{-8}$ M 20-HETE in the NS rats but had no effect on these responses in the HS rats.

Figure 6 illustrates the effect of KCa channel inhibition with IbTx on arteriolar responses to increased oxygen availability. In both dietary groups, the magnitude of oxygen-induced arteriolar constriction was markedly reduced by IbTx, with the values for NS and HS rats no longer being significantly different in the presence of IbTx.

There was no significant difference in arteriolar responsiveness to ANG II between dietary groups (Fig. 7, top). In the presence of DDMS, these responses were consistently decreased in the NS group. This effect of DDMS was noticeably less in the HS group, with significant inhibition observed only at the lower concentration of ANG II. In contrast, arteriolar responses to NE were greater in HS rats than in NS rats (Fig. 7, bottom), but 20-HETE inhibition with DDMS had no consistent effect on these responses in either group, except for a modest reduction in responses to the higher concentration of NE in HS rats. As shown in Fig. 8, KCa channel inhibition with IbTx significantly increased arteriolar responses to NE in NS rats but not in HS rats (Fig. 8).

DISCUSSION

The major findings of this study are as follows. 1) Long-term (4 wk) ingestion of a high-salt diet attenuates the arteriolar constriction and blood flow reduction associated with increased oxygen availability in skeletal muscle. 2) Inhibition of CYP450
4A ω-hydroxylase decreases these oxygen-induced responses in rats fed a normal diet but not in rats fed a high-salt diet. 3) Arteriolar responses to exogenous 20-HETE are reduced and no longer sensitive to K$_{Ca}$ channel inhibition in rats fed high salt. 4) Arteriolar responses to ANG II are similar in rats fed normal and high-salt diets, but CYP450 4A ω-hydroxylase inhibition is less effective in reducing these responses in rats fed high salt. These findings suggest that, independent of any change in blood pressure, high dietary salt intake can impair arteriolar responsiveness to oxygen and that this effect is due to a reduction in the contribution of endogenous 20-HETE to the arteriolar constriction.

In a recent study (36), we reported that ingestion of a high-salt diet leads to a blunting of the arcade arteriole dilation that accompanies contraction of rat spinotrapezius muscle. We have also obtained evidence that H$_2$O$_2$, generated by the contracting muscle fibers, is one of the key vasoactive molecules that contribute to this arteriolar dilation (35). However, the role of H$_2$O$_2$ in this dilation is not diminished by high-salt intake, suggesting that the salt-induced decrease in functional dilation must be due to a reduction in the influence of some other dilator stimulus. A decrease in local oxygen levels can also contribute to arteriolar dilation during muscle contraction (7, 17, 41), either directly if there is a fall in arteriolar wall PO$_2$ or indirectly via vasoactive mediators produced when PO$_2$ declines in nearby parenchymal cells or paired venules (27, 43). Because the blunting of functional dilation in salt-fed rats could reflect a reduced arteriolar responsiveness to such changes in PO$_2$, one aim of this study was to examine the effects of a high-salt diet on arteriolar responses to oxygen. We found no significant difference in resting tissue PO$_2$ between NS and HS rats, which is consistent with the similar levels of resting arteriolar tone and blood flow in the two groups (Table 1).

Elevation of superfusate oxygen also increased tissue PO$_2$ by the same amount in both groups, suggesting that the stimulus for arteriolar constriction was also similar in NS and HS rats. The size of the sensors we used precluded direct PO$_2$ measurements on the arteriolar wall, but an earlier study using smaller oxygen-sensitive microelectrodes in the same preparation studied here indicates that an increase in superfusate oxygen from 0% to 10% increases arcade arteriole wall PO$_2$ from 42 ± 8 mmHg to 66 ± 6 mmHg, values considered to be within the normal physiological range (27). In the current study, the increase in tissue oxygen levels decreased arcade arteriole diameter and flow in rats fed both diets, but these responses were significantly less in rats fed high salt for 4 wk (Fig. 1). In light of previous findings that oxygen-induced arteriolar constriction is similar in cremaster muscle of rats fed 4% vs. 0.4% salt for 3 days (13), our findings suggest that, when compared with larger extraparenchymal resistance vessels (47, 48), a more prolonged period of high salt intake is required to alter the oxygen responsiveness of smaller intramuscular arterioles.

In rat cremaster muscle, oxygen-induced arteriolar constriction is largely mediated by the CYP450 4A ω-hydroxylase metabolite 20-HETE (12, 13, 19, 25, 34). An increase in this contribution of 20-HETE to abnormal arteriolar responses in normotensive rats fed high salt, a second aim of this study was to determine whether oxygen-induced arteriolar constriction in rat spinotrapezius muscle is also dependent on CYP450 4A ω-hydroxylase activity and, if so, to determine whether high salt intake alters the importance of this pathway. Under normal conditions, oxygen-induced constriction of spinotrapezius muscle arterioles was greatly reduced following inhibition of 20-HETE formation with either 17-ODYA or DDMS (Fig. 2). This is consistent with earlier findings in rat and hamster cremaster muscle (12, 13, 19, 25, 34) and hamster retractor muscle (34) and strongly suggests that 20-HETE plays a universal role in mediating arteriolar oxygen responsiveness in skeletal muscle.

Fig. 3. Top: Western blot expression of 51-kDa cytochrome P-450 4A (CYP4A) protein in spinotrapezius muscle of rats fed NS and HS diets. Bottom: mean CYP4A band intensities expressed as a percentage of loading control protein (GAPDH) intensity. n, Number of samples (rats).

Fig. 4. Changes in arteriolar diameter with 20-HETE application in rats fed NS and HS diets. For NS, n = 8 vessels for the 1 × 10$^{-9}$ M and 1 × 10$^{-8}$ M applications and 9 vessels for the 5 × 10$^{-8}$ M application. For HS, n = 8 vessels for the 1 × 10$^{-9}$ M and 1 × 10$^{-8}$ M applications and 10 vessels for the 5 × 10$^{-8}$ M application. *P < 0.05 vs. NS.
regardless of species or muscle type. Our Western analysis indicates that, in both dietary groups, CYP450 4A \( \omega \)-hydroxylase is expressed in the arteriolar wall (data not shown) as well as in skeletal muscle fibers (Fig. 3), which is consistent with previous findings in rat cremaster muscle (25) and implies that arteriolar responses to a change in oxygen availability can be mediated by 20-HETE released from the arterioles themselves and/or from the surrounding skeletal muscle fibers.

In contrast with rats fed the normal diet, neither 17-ODYA nor DDMS had an effect on oxygen-induced arteriolar constriction or the accompanying flow reduction in rats fed high salt (Fig. 2), suggesting a loss of 20-HETE’s contribution to these responses. This could be due to 1) a decrease in CYP450 4A \( \omega \)-hydroxylase expression or activity, leading to decreased 20-HETE formation, and/or 2) a reduction in vascular smooth muscle responsiveness to the 20-HETE that is formed. With respect to the first possibility, we found no difference between dietary groups in overall skeletal muscle expression of CYP450 4A \( \omega \)-hydroxylase (Fig. 3), but because of the technical limitations discussed earlier, we are not able to rule out the possibility of salt-dependent differences in enzyme expression within the microvessels themselves. However, our findings clearly support the second possibility, in that arteriolar constrictor responses to exogenous 20-HETE are greatly reduced in rats fed high salt (Fig. 4). To our knowledge, this effect of a high-salt diet on arteriolar smooth muscle responsiveness to 20-HETE has not been previously evaluated. In contrast with our findings, Frisbee et al. (13) did not find a reduced contribution of 20-HETE to oxygen-induced constriction of skeletal muscle arterioles in rats fed a high-salt diet for 3 days, again suggesting that a longer period of high-salt intake is necessary to influence this mechanism in the arterioles than in upstream arteries.

Short-term and long-term high salt intake (from 3 days to as long as 8 wk) can also impair the dilation of small gracilis muscle feed arteries to reduced oxygen availability (14, 30, 47). Such dilation is also mediated partly through a change in 20-HETE production—in this case a decrease from control levels (16, 19). Because the general reduction in arteriolar smooth muscle responsiveness to 20-HETE that we found should reduce the overall slope of the inverse relationship between \( P_{O_2} \) and arteriolar diameter, this characteristic could account for the smaller dilation to reduced oxygen as well as the smaller constriction to increased oxygen. Thus our current findings are also consistent with the hypothesis that reduced arteriolar dilation during skeletal muscle contraction in salt-fed rats is due, in part, to reduced arteriolar oxygen responsiveness (36).

We found that inhibition of large-conductance KC\(_a\) channels with TEA or IbTx decreased resting arteriolar diameters by a similar amount in both dietary groups, implying 1) that these channels are sufficiently active under resting conditions to influence resting smooth muscle membrane potential and therefore modulate contractile activity and 2) that this channel activity is not affected by dietary salt intake. An influence of KC\(_a\) channel activity on the resting tone of gracilis muscle arterioles has also been reported by this laboratory and others (5, 44), but these findings do not extend to arterioles in all skeletal muscles or in some other vascular beds. For example, arteriolar smooth muscle KC\(_a\) channel activity appears to be silent under resting conditions in rat and hamster cremaster muscle (23, 31) and rat cerebral cortex (39). These vessel-to-vessel differences in the steady-state activity of KC\(_a\) channels at normal vascular tone could be due to differences in the Ca\(^{2+}\) set point of these channels, as suggested by Jackson and Blair (23).

20-HETE increases vascular tone, in part, by inhibiting the activity of vascular smooth muscle KC\(_a\) channels, leading to membrane depolarization and a subsequent influx of calcium.
through L-type voltage-sensitive channels (1, 18, 52). When these channels were inhibited by TEA or IbTx before 20-HETE application, the arteriolar constriction to 20-HETE was dramatically reduced in rats fed normal salt but not in rats fed high salt (Fig. 5). This suggests that the reduced vasoconstriction to exogenous 20-HETE in salt-fed animals is due to a loss of that part of the response linked to reductions in KCa channel activity. It seems unlikely that this is due to a general resistance of KCa channels to inhibition, since previous studies have documented that K⁺ channel function and resting vascular smooth muscle membrane potential are not altered by high salt intake in normotensive rats (14, 32), and, as stated above, we found no difference between dietary groups in the effects of TEA or IbTx on resting arteriolar tone. Instead, high salt intake may lead to the disruption of some 20-HETE-triggered event in vascular smooth muscle that precedes KCa channel inhibition. The details of this part of the 20-HETE signaling pathway are not well understood. Although a vascular smooth muscle 20-HETE receptor has not yet been identified, its existence is suggested by the finding that structural analogs of 20-HETE can inhibit arteriolar constriction (1). The postreceptor events leading to KCa channel inhibition apparently involve activation of a protein kinase C and a tyrosine kinase and possibly phosphorylation of channel residues by the small G protein Raf (42). Theoretically, high salt intake could disrupt any of these events or even reduce receptor affinity for 20-HETE. Further studies will be necessary to provide these important details.

In rats fed the normal diet, the large decrease in oxygen-induced constriction following IbTx exposure (Fig. 6) is consistent with our data suggesting that there is a predominant role for 20-HETE in these responses (Fig. 2) and that 20-HETE increases arteriolar tone largely by modulating KCa channel activity (Fig. 5). As also shown in Fig. 6, we found that IbTx also reduces arteriolar responses to oxygen in rats fed high salt, where there is no apparent contribution of 20-HETE to the...
constriction (Fig. 2). This suggests that the 20-HETE-independent pathways through which oxygen can increase vascular tone also involve some modulation of K_{Ca} channel activity. In this context, it is worth noting earlier evidence that oxygen can also induce arteriolar constriction in rat spinotrapezius muscle by decreasing the local production of nitric oxide (40), which can influence vascular smooth muscle tone by changing membrane K_{Ca} channel activity (4, 8).

In this study, we found that ANG II induced similar arteriolar constrictions in both dietary groups (Fig. 7, top), which is consistent with an earlier report that responses of cremaster muscle arterioles to ANG II are not altered in rats fed 4% salt for the same duration as in the current study (49). However, gracilis muscle resistance arteries from rats placed on the same high-salt regimen do exhibit enhanced ANG II responses, possibly due to an upregulation of ANG II receptors in response to the salt-induced reduction in circulating ANG II (45). If so, then the absence of such a change in the responsiveness of downstream arterioles could reflect a difference between the arterioles and more proximal arteries in the relative distribution of ANG II receptor subtypes (29). Vascular smooth muscle constriction to ANG II is partly mediated by 20-HETE formation in rat renal interlobular arteries and afferent arterioles (3, 9, 20) and in rat cremaster muscle arterioles (8). Consistent with these findings, 20-HETE inhibition reduced arteriolar constriction to ANG II in both of the groups we studied, although this effect was decreased in high-salt rats (Fig. 7). The smaller contribution of 20-HETE to this constriction in the salt-fed rats could be related to the reduction in arteriolar smooth muscle responsiveness to 20-HETE.

Although 20-HETE also partially mediates the contractile effect of NE in rat mesenteric resistance arteries (46) and rabbit aortic smooth muscle cells (24), 20-HETE inhibition did not significantly reduce NE-induced arteriolar constriction in the spinotrapezius muscle of rats fed the normal diet (Fig. 7), suggesting that there could be differences in the mechanism of adrenergic constriction between arterioles and upstream arterioles. In contrast with the ANG II responses described above, we found that arteriolar responses to NE were consistently enhanced in rats fed high salt, and the significant reduction of these responses to the higher level of NE by DDMS suggests that this enhancement is at least partially due to the addition of a 20-HETE component to the response. This is surprising in light of this study’s main finding that oxygen-induced arteriolar constriction is reduced in salt-fed rats due to a decrease in 20-HETE’s contribution to those responses. Neither the cause of this dichotomy nor its physiological significance is immediately clear; further studies will be necessary to gain more insight into these issues. We found no difference between groups in resting arteriolar tone (Table 1), but such enhanced responsiveness to NE in the salt-fed rats could theoretically result in a greater increase in the adrenergic tone of these vessels during periods of increased sympathetic nervous outflow.

Although 20-HETE apparently does not contribute to NE-induced arteriolar constriction in NS rats, we found that IbTx had no effect on arteriolar responses to NE in HS rats, implying that NE does not normally stimulate this feedback pathway in salt-fed rats, possibly because the generation of 20-HETE in these vessels prevents K_{Ca} channels from being activated (Fig. 7).

In conclusion, the present study demonstrates that 20-HETE plays an important role in the oxygen-induced constriction of spinotrapezius muscle arterioles. In contrast with previous findings in rats fed high salt for 3 days, a 4- to 5-wk period of high salt intake leads to an attenuation of this response to oxygen, largely through a reduction in the intrinsic responsiveness of arteriolar smooth muscle to 20-HETE. These findings also raise the possibility that reduced arteriolar dilation during skeletal muscle contraction in salt-fed rats could be due to impaired arteriolar oxygen sensitivity.

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REFERENCES


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