Integration of hypoxic dilation signaling pathways for skeletal muscle resistance arteries

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Frisbee, Jefferson C., Kristopher G. Maier, John R. Falck, Richard J. Roman, and Julian H. Lombard. Integration of hypoxic dilation signaling pathways for skeletal muscle resistance arteries. Am J Physiol Regulatory Integrative Comp Physiol 283: R309–R319, 2002. First published March 29, 2002; 10.1152/ajpregu.00741.2001.—Mediator contributions to hypoxic dilation of rat gracilis muscle resistance arteries were determined by measuring dilation, vascular smooth muscle hyperpolarization, and metabolite production after incremental hypoxia. Nitric oxide (NO) synthase inhibition abolished responses to mild hypoxia, whereas COX inhibition impaired responses to more severe hypoxia by 77%. Blocking 20-hydroxyeicosatetraenoic acid (20-HETE) impaired responses to moderate hypoxia. With only NO systems intact, responses were maintained with mild hypoxia (88% normal) mediated via KCa channels. When only COX pathways were intact, responses to moderate-severe hypoxia were largely retained (79% of normal) mediated via KATP channels. Vessel responses to moderate hypoxia were retained with only 20-HETE systems intact mediated via KCa channels. NO production increased 5.6-fold with mild hypoxia; greater hypoxia was without further effect. With increased hypoxia, 20-HETE levels fell to 40% of control values. 6-keto-PGF1α, levels were not altered with mild hypoxia, but increased 4.6-fold with severe hypoxia. These results suggest vascular reactivity to progressive hypoxia represents an integration of NO production (mild hypoxia), PGI2 production (severe hypoxia), and reduced 20-HETE levels (moderate hypoxia).

Throughout the body, microvessels use a complex array of integrative mechanisms to regulate their tone in response to a wide variety of vasoactive stimuli and physiological processes (10, 13). Of these numerous contributing factors, investigation into the role of oxygen in mediating vascular tone has produced a considerable body of literature, albeit one with little consensus on mechanisms underlying the observations. As such, determining the manner in which these processes integrate to produce net responses of microvessels to altered oxygen tension remains an elusive target.

With specific regard to skeletal muscle microvessel reactivity to altered Po2, results from previous studies have suggested that either endothelium-derived nitric oxide (NO; 14, 22), endothelium-derived prostacyclin (PGI2; 5–8, 17–20), or smooth muscle-derived 20-hydroxyeicosatetraenoic acid (20-HETE; 6, 7, 11, 16) may be the predominant mediator of this process, although a recent study (6) also suggested that endothelium-derived epoxyeicosatrienoic acids (EETs) may also play a contributing role. Despite the fact that these studies encompass skeletal muscle microvessels ranging from resistance arteries to distal arterioles and that vessels from different skeletal muscles studied under different conditions have been employed for these studies, it can be difficult to reconcile the wide divergence in proposed mediators of vessel dilation to reduced oxygen tension. It is clearly necessary to systematically dissect, within specific vascular segments, the pathways contributing to O2-induced alterations in microvessel tone. The present study was performed to determine the interaction and relative contribution of the predominant mediators of hypoxic dilation to the relaxation and vascular smooth muscle (VSM) membrane hyperpolarization of resistance arteries serving rat gracilis muscle during exposure of these vessels to incremental levels of hypoxia similar to those that may be encountered under a variety of in vivo conditions. We hypothesize that responses of skeletal muscle resistance arteries to incremental, graded reductions in Po2 do not reflect a single mechanism, but rather are the integrated effect of multiple mechanisms acting through the range of hypoxia to contribute to the dilation and VSM hyperpolarization that occur in these vessels’ response to reduced oxygen tension. It is important to emphasize that the purpose of the present study was not to determine which signaling pathways contribute to the dilation of skeletal muscle microvessels in response to reduced Po2, as this has been investigated in considerable detail in the previous studies cited above. Determining the interplay of significant mediators of hypoxia-in
duced alterations in vascular tone in these vessels represents an important avenue of investigation, as they lie immediately proximal to the microcirculation per se and play a critical role in regulating the flow of blood through distal arterial networks (4).

**MATERIALS AND METHODS**

**Animals.** All experiments employed 9- to 14-wk-old male Sprague-Dawley rats (weight = 319 ± 13 g; mean arterial pressure = 109 ± 6.4 mmHg) maintained on standard rat chow and tap water ad libitum. Rats were housed in an animal care facility at the Medical College of Wisconsin that is accredited by the American Association for the Accreditation of Laboratory Animal Care and all protocols received prior approval from the Institutional Animal Care and Use Committee. Rats were anesthetized with an injection of pentobarbital sodium (60 mg/kg ip), and a carotid artery was cannulated for determination of arterial pressure immediately before isolation of vessels for study.

**Preparation of isolated vessels.** The small muscular branch of the femoral artery supplying the gracilis muscle was removed from the anesthetized rat, taking care to minimize vessel stretching and to handle arteries by their surrounding connective tissue only. Arteries were placed in a heated chamber (37°C) that allowed the lumen and exterior of the vessel to be perfused and superfused, respectively, with physiological salt solution (PSS) from separate reservoirs. The PSS used in these experiments was equilibrated with a 21% O2, 5% CO2, and 74% N2 gas mixture and had the following composition (mM): 119 NaCl, 4.7 KCl, 1.17 MgSO4, 1.6 CaCl2, 1.18 NaH2PO4, 24 NaHCO3, 0.026 EDTA, and 5.5 glucose. Vessels were cannulated at both ends with glass micropipettes and were secured to the inflow and outflow pipettes using 10–0 nylon suture. Any side branches were ligated with a single strand teased from 6–0 silk suture. The inflow pipette was connected to a reservoir perfusion system that allowed the intraluminal pressure and luminal gas concentrations to be controlled. Vessel diameter was measured using television microscopy and an on-screen video micrometer (15).

Arteries were extended to their approximate in situ length and were equilibrated at 80% of the animal’s mean arterial pressure (88 ± 4.9 mmHg) to approximate in vivo perfusion pressure (15). Under these conditions, vessels experienced a wall shear stress ranging from 1.5 to 2.1 dynes/cm2. Vessels that did not demonstrate both a functional endothelium and active tone at rest (assessed by a brisk dilation in response to 10−6 M acetylcholine) were discarded. Active tone at the equilibration pressure was calculated as (AD/Dmax)×100, where AD is the diameter increase from rest in response to Ca2+-free PSS, and Dmax is the maximum diameter measured at the equilibration pressure in Ca2+-free PSS. Active tone for vessels in the present study averaged 35.5 ± 2.2%.

**Measurement of VSM membrane potential.** VSM transmembrane potential was measured with a high-impedance amplifier and glass microelectrodes (40–80 MΩ impedance) filled with 3 M KCl. Criteria for successful implantation included an abrupt drop to a steady level of transmembrane potential for a minimum of 5 s and a rapid return to baseline after removal of the electrode from the cell. Five measurements were made under each condition (i.e., in response to each challenge), and the results were averaged to obtain the final value of transmembrane potential for that vessel under each experimental condition (8, 17).

**Assessment of vessel dilation and VSM hyperpolarization to hypoxia.** Arterial dilation and VSM hyperpolarization in response to incremental hypoxia were determined at the equilibrium pressure for each vessel. For each artery, the O2 content of the superfusate/perfusate equilibration gas was reduced from 21% O2 (145–150 mmHg at the vessel) to either 15% O2 (115–120 mmHg at the vessel), 10% O2 (80–85 mmHg at the vessel), 5% O2 (60–65 mmHg at the vessel), or 0% O2 (35–40 mmHg at the vessel). All gas equilibration mixtures contained 5% CO2 and balance N2. For the determination of oxygen tension experienced by the isolated vessel, oxygen electrodes were placed within the vessel chamber and at the opening of the perfusate cannula leading into the vessel tension (5). Vessel diameter and VSM transmembrane potential measurements were taken after 30 min at each O2 level, and the imposed levels of hypoxia were randomized for all experiments. All measurements were taken under no-flow conditions, with the intraluminal pressure within the vessel at 80% of the mean arterial pressure for the individual animal (see above).

**Removal of the vascular endothelium.** The endothelium of isolated vessels was removed via air bolus perfusion (7). Endothelium denudation procedures were deemed successful when dilation in response to 10−6 M acetylcholine was eliminated, whereas responses to 10−6 M sodium nitroprusside were unaltered.

**Inhibition of cytochrome P-450 systems.** To assess the role of arachidonic acid epoxidation in contributing to hypoxic dilation of arteries, cytochrome P-450 (CP450) epoxygenases were inhibited with the suicide substrate N-methylsulfonyl-6-(2-proparglyoxyphenyl)hexanoic acid (MS-PPOH; 2×10−5 M; 6, 12). Direct measurements of biochemical metabolites in rat renal microsomes indicated that MS-PPOH is a selective inhibitor of arachidonic acid epoxidation, with minimal effects on the ω-hydroxylation reaction of arachidonic acid (24). To evaluate the role of 20-HETE in regulating arterial dilation to reduced PO2, these responses were determined after addition of the synthetic compound 20-hydroxyeicosatetraenoic acid [6(Z),15(Z)-20-HEDE; 10−6 M; 1, 7], a potent competitive antagonist of the actions of 20-HETE, to the tissue bath.

**Inhibition of potassium channels.** To determine the contribution of adenosine triphosphate-sensitive potassium channels (KATP) and large conductance Ca2+-activated potassium channels (KCa) to the response of gracilis arteries to incremental hypoxia, these channels were inhibited with glibenclamide (10−6 M; 7, 17) or iberiotoxin (10−7 M; 3, 7, 9), respectively, in the vessel chamber. Previous studies in our laboratory indicated that these concentrations of glibenclamide and iberiotoxin effectively block responses of resistance arteries to the prostacyclin analog iloprost and 20-HETE, respectively (5, 7).

**Inhibition of prostanooid and NO production.** To assess the role of prostanooid or NO release from NADPH oxidase to arterial hypoxia in regulating the response of isolated arteries to reduced PO2, the cyclooxygenase inhibitor indomethacin (10−6 M; 7, 17) or the NO synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME; 10−4 M; 7, 21) was added to the vessel bath to inhibit prostanooid or NO production, respectively.

**Determination of NO production.** In a separate series of experiments, the generation of NO from gracilis muscle resistance arteries was determined using an amperometric NO sensor (ISO-NOP200 Mark II; World Precision Instruments). The sensor was calibrated as described by the manufacturer by generating known concentrations of NO in solution through the use of a 10−6 M solution of the NO donor.
S-nitroso-N-acetylpenicillamine (SNAP). The calibration curve describing the sensitivity of the sensor to NO was determined using four dilutions of the SNAP solution and the measured amperage from the NO sensor. With this use of basic regression, this allowed for the determination of a linear relationship between NO concentration within the diluted SNAP solutions and the current measured by the sensor ($r^2 = 0.983$).

Vessels from anesthetized rats were surgically removed and were incubated in glass vials containing 1 ml of PSS equilibrated with 21% O$_2$. After 30 min in 21% O$_2$, the PSS equilibration gas was either kept at 21% O$_2$ or was switched to a different (randomized) O$_2$ content (described above) for an additional 30 min. Over the final 5 min of this second period, NO values were determined using the sensor, and the final level of NO concentration within the solution produced from the vessels represents the time-averaged value over this 5-min period. For each determination of NO production, $n = 12$ gracilis arteries pooled from six rats under each of five oxygen levels. Final values represent the data collected from six groups of rats (total = 36 rats).

**Determination of prostacyclin production.** The production of PGF$_2$ by gracilis muscle resistance arteries in response to incremental hypoxia was assessed as described previously (15), with minor modification. Briefly, vessels were removed from anesthetized rats and were pooled in the manner described above. Vessels were incubated in glass vials in 1 ml of PSS for 30 min under control conditions (21% O$_2$), after which the equilibration gas was switched to one of the other four mixtures (randomized) or remained at 21% O$_2$ for an additional 30 min. After the second 30-min period, the PSS was removed from the incubation chamber, frozen in liquid N$_2$, and stored at −80°C. Immediately, 1 ml of fresh PSS (equilibrated with 21% O$_2$) was added to the pooled vessels in the glass vial and the procedure was repeated for the subsequent oxygen level. PGF$_2$ release by vessels under the different oxygen levels was assessed in the Department of Physiology Biochemical Core Facility at the Medical College of Wisconsin by measuring the level of 6-keto-prostaglandin F$_1$ (6-keto-PGF$_{1a}$), the stable metabolite of PGF$_2$, in the incubation medium. Measurements were made using a commercially available EIA kit purchased from Cayman Chemical (Ann Arbor, MI). The numbers and grouping of microvessels and rats for these experiments were identical to those outlined for the Determination of NO production.

**Determination of 20-HETE production.** In the final series of experiments, 20-HETE production from gracilis arteries was determined using a fluorescence HPLC assay (18). Briefly, vessels were removed, pooled, and exposed to the different O$_2$ levels as described above. After exposure to either 21% O$_2$ or to one level of hypoxia, vessels were snap-frozen in liquid N$_2$. Subsequently, the pooled arteries were homogenized, acidic lipids were extracted with ethyl acetate, and the samples were dried under nitrogen. The fatty acids were fluorescently labeled and separated using reverse-phase HPLC, as fully described previously (18). For each measurement of 20-HETE production, $n = 4$ gracilis arteries pooled from two rats under each of the five oxygen levels. Final values represent the data collected from four groups of rats (total = 40 rats).

**Data and statistical analyses.** All data are presented as means ± SE. For the present study, the mechanical and electrical responses of vessels in response to incremental hypoxia under control conditions (i.e., endothelium-intact vessels with no pharmacological intervention) are assumed to represent normal responses to reduced PO$_2$ within those vessels. For the subsequent data presentation, vessel mechanical and electrical responses to each level of reduced PO$_2$ under an experimental intervention (e.g., treatment with L-NAME) have been normalized to responses determined in untreated vessels within that subgroup. As a result, vascular responses to each reduction in PO$_2$ in the experimental groups represent a percentage of the original control response to that level of hypoxia. For example, if dilation of a vessel under control conditions in response to a given level of hypoxia is 10 μm and treatment of the vessel with a pharmacological agent reduces this response to 4 μm, 40% of the normal reactivity of the vessel to hypoxia is retained after treatment with the drug. Statistically significant differences in resting diameter and VSM transmembrane potential of the vessel were determined using a one-way ANOVA, whereas responses to reduced PO$_2$ and metabolite production with incremental hypoxia were determined using ANOVA or repeated-measures ANOVA, as appropriate. All analyses employed Tukey’s post hoc test. For all analyses, $P < 0.05$ was taken to be statistically significant.

**RESULTS**

Data describing the diameter and VSM transmembrane potential of isolated arteries under 21% O$_2$ for the conditions of the present study are presented in Table 1. Treatment of arteries with glibenclamide or iberiotoxin caused a significant constriction of the vessels and a significant depolarization of the VSM membrane compared with values under control conditions. Combined application of L-NAME and indomethacin also caused a vasoconstriction and VSM depolarization from control values.

Preliminary experiments performed in our laboratory indicated that application of the prostacyclin analog iloprost (10$^{-9}$ g/ml) and the NO donor 6-(2-hydroxy-1-methyl-2-nitrosohydrodrazino)-N-methyl-1-hexanamine (10$^{-6}$ M) caused a significant hyperpolarization of the VSM cell.

<table>
<thead>
<tr>
<th>Treatment Condition</th>
<th>Diameter, μM</th>
<th>VSM $E_{m}$, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>124 ± 1.0</td>
<td>−41.0 ± 1.6</td>
</tr>
<tr>
<td>Endothelium removed</td>
<td>120 ± 3.0</td>
<td>−38.5 ± 1.9</td>
</tr>
<tr>
<td>+10$^{-4}$ M L-NAME</td>
<td>118 ± 2.2</td>
<td>—</td>
</tr>
<tr>
<td>+10$^{-6}$ M indomethacin</td>
<td>123 ± 3.2</td>
<td>—</td>
</tr>
<tr>
<td>+2×10$^{-5}$ M MS-PPOH</td>
<td>125 ± 3.2</td>
<td>—</td>
</tr>
<tr>
<td>+10$^{-8}$ M 6(Z),15(Z)-20-HEDE</td>
<td>124 ± 3.1</td>
<td>—</td>
</tr>
<tr>
<td>+10$^{-7}$ M iberiotoxin</td>
<td>111 ± 2.4*</td>
<td>−26.5 ± 1.2*</td>
</tr>
<tr>
<td>+10$^{-6}$ M glibenclamide</td>
<td>115 ± 2.2*</td>
<td>−28.1 ± 1.5*</td>
</tr>
<tr>
<td>+10$^{-6}$ indomethacin; 10$^{-6}$ M 6(Z),15(Z)-20-HEDE</td>
<td>122 ± 2.8</td>
<td>−37.8 ± 1.6</td>
</tr>
<tr>
<td>+10$^{-4}$ L-NAME; 10$^{-6}$ M</td>
<td>121 ± 2.4</td>
<td>−37.2 ± 1.5</td>
</tr>
<tr>
<td>+10$^{-6}$ M indomethacin; 10$^{-4}$ L-NAME</td>
<td>113 ± 3.0*</td>
<td>−32.8 ± 1.3*</td>
</tr>
<tr>
<td>Ca$^{2+}$-free PSS</td>
<td>194 ± 2.1*</td>
<td>—</td>
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</table>

Values are means ± SE. For measurements of microvessel diameter under treated conditions, $n = 6–10$ vessels; for measurements of vascular smooth muscle (VSM) transmembrane potential ($E_{m}$) under treated conditions, $n = 5$ vessels. L-NAME, N$^\omega$nitro-L-arginine methyl ester; MS-PPOH, N-methylsulfonyl-6-(2-propargyloxyphenyl hexanoic acid; HEDE, 20-hydroxyeicos-6(Z),15(Z)-dienoic acid; PSS, physiological salt solution. *$P < 0.05$ vs control.
membrane of isolated gracilis arteries from $-41.2 \pm 0.5$ mV to $-57.4 \pm 1.8$ mV and $-59.3 \pm 1.8$ mV, respectively.

Mechanical and electrical responses of microvessels during incremental hypoxia. Figure 1 presents data describing the dilation of isolated gracilis arteries (Fig. 1A) and the hyperpolarization of the VSM membrane of these vessels (Fig. 1B) after incremental reductions in PO$_2$ under control conditions (i.e., intact vessels with no pharmacological agent present). The progressive reductions in oxygen tension caused significant increases in the diameter and significant hyperpolarizations of the VSM membrane of isolated gracilis arteries. Figure 1C presents the changes in VSM transmembrane potential vs. arterial diameter in response to incremental hypoxia. These data suggest that hypoxia-induced changes in gracilis artery diameter are primarily a function of alterations in VSM transmembrane potential.

Figure 2 presents data describing vessel dilation (Fig. 2A) and hyperpolarization of the VSM cell membrane (Fig. 2B) in response to incremental hypoxia after removal of the vascular endothelium. Endothelium denudation nearly abolished arterial responses to graded reductions in PO$_2$, although vessels did retain some reactivity to low oxygen tension as the severity of hypoxia worsened. This is indicated by the persistence of 15–25% of the normal mechanical and electrical responses of the vessels to severe hypoxia (5% and 0% O$_2$) after removal of the endothelium.

Data describing arterial dilation in response to graded hypoxia after inhibition of specific enzyme systems are presented in Fig. 3. Inhibition of NO synthase with l-NAME abolished arterial dilation in response to mild hypoxia (15% O$_2$), but had little impact on vessel dilation to more severe hypoxia (5% and 0% O$_2$). In contrast, inhibition of COX enzymes had no effect on arterial dilation to 15% O$_2$, but strongly impaired vessel relaxation to more severe hypoxia. Treatment of the vessel with 6(Z),15(Z)-20-HEDE had no effect on vessel dilation with mild reductions in PO$_2$, although arterial dilation in response to moderate (10% O$_2$) and severe hypoxia was significantly attenuated. Inhibition of CP450 epoxygenases with MS-PPOH had no effect on arterial dilation at any level of hypoxia.

Data describing arterial dilation (Fig. 4A) and VSM membrane hyperpolarization (Fig. 4B) after treatment of vessels with multiple pharmacological agents are presented in Fig. 4. During treatment of arteries with indomethacin and 6(Z),15(Z)-20-HEDE (leaving only NO synthase pathways intact), vessel dilation and VSM hyperpolarization in response to mild hypoxia (15% O$_2$) were unaltered, although the responses to increasing hypoxia were severely impaired. Treating vessels with l-NAME and 6(Z),15(Z)-20-HEDE (leaving only COX pathways intact) abolished microvessel responses to mild hypoxia, but had little effect on the responses to more severe hypoxia. Finally, treating vessels with l-NAME and indomethacin (leaving only 20-HETE systems intact) abolished vessel responses to mild hypoxia and severely impaired the responses of the arteries to more severe hypoxia. Combined treatment of vessels with all three inhibitors (l-NAME,
indomethacin, and 6(Z),15(Z),20-HEDE] abolished the reactivity of gracilis arteries to reduced P O2.

The effects of treating vessels with K+ channel antagonists on vasodilation (Fig. 5A) and VSM hyperpolarization (Fig. 5B) in response to reduced P O2 are presented in Fig. 5. Blockade of KATP channels with glibenclamide had no effect on microvessel responses to mild hypoxia, but severely inhibited the response of arteries to more severe hypoxia. In contrast, inhibition of KCa channels with iberiotoxin abolished arterial reactivity to mild hypoxia, but only modestly impaired responses through the remainder of the imposed range of hypoxia. Combined treatment of vessels with both K+ channel antagonists eliminated all reactivity of isolated gracilis arteries to reduced P O2 (data not shown).

Metabolite production from arteries in response to incremental hypoxia. Under control conditions (21% O2), pooled gracilis arteries had an NO production of 4.5 ± 1.0 nmol/mg wet vessel weight, a 6-keto-PGF1α production of 13.6 ± 1.9 pg/mg wet vessel weight, and a 20-HETE content of 2.0 ± 0.2 ng/mg wet vessel weight. Representative chromatograms from the HPLC determination of vascular 20-HETE content under conditions of 0% O2 (Fig. 6A) and 21% O2 (Fig. 6B) are presented in Fig. 6. Figure 7 presents data describing changes in the production of NO, 6-keto-PGF1α, and 20-HETE in isolated pooled arteries after graded reductions in PO2. With mild hypoxia (15% O2), NO release from vessels increased significantly from levels measured under control conditions (Fig. 7A), but NO production exhibited no further increase with more severe hypoxia. PGI2 release from vessels, estimated from its stable breakdown product, 6-keto-PGF1α, was elevated only with moderate to severe reductions in PO2 (Fig. 7B). 20-HETE production by vessels fell parallel to PO2 (Fig. 7C), although the majority of this effect occurred between 15% O2 and 5% O2.

Interaction of vasoactive metabolites and potassium channels in arteries with incremental hypoxia. Data describing the interaction of KATP channels (Fig. 8A) and KCa channels (Fig. 8B) with the predominant mediators of hypoxic dilation in isolated arteries are presented in Fig. 8. Similar to data presented in Fig. 5, inhibition of KATP channels with glibenclamide had no effect on arterial dilation in response to mild hypoxia, but severely impaired this response with increasing hypoxia. Combined application of glibenclamide with the NO synthase inhibitor L-NAME eliminated vessel dilation in response to mild hypoxia, but had minimal
further impact on the dilation of the vessels with increased severity of hypoxia. Treatment of arteries with glibenclamide and either indomethacin (to inhibit COXs) or 6(Z),15(Z),20-HEDE (to block the effects of 20-HETE) had minimal effect on the dilation of vessels in response to incremental hypoxia compared with arteries treated with glibenclamide alone. Also consistent with data presented in Fig. 5, treatment of vessels with iberiotoxin (to block KCa channels) abolished vessel dilation in response to mild hypoxia and significantly impaired this response with moderate to severe hypoxia (Fig. 8B). Combined application of iberiotoxin with either l-NAME or 6(Z),15(Z)-20-HEDE had minimal additional effect on the response of vessels to incremental hypoxia beyond that determined in iberiotoxin-treated vessels alone. In contrast, treatment of vessels with both iberiotoxin and indomethacin completely eliminated the dilation of the vessels in response to reduced PO2.

**DISCUSSION**

Given that investigations of mechanisms for vascular reactivity to hypoxia have demonstrated considerable heterogeneity regarding the signaling pathways involved, the purpose of the present study was to begin to assemble the predominant mediators of hypoxic dilation of skeletal muscle microvessels into a more in-
integrated framework. Previous studies suggested that release of endothelium-derived NO (14, 22), prostacyclin (5–8, 15, 17, 19, 20), or EETs (6) mediates the dilation of rat skeletal muscle microvessels in response to large, discrete reductions in PO2, with additional evidence suggesting that a reduction in vascular levels of 20-HETE (6, 7, 11, 16) could also play a role in mediating this response. However, many of these studies suggest that a specific mediator dominates this response, with little contribution from other signaling pathways toward determining the net reactivity of skeletal muscle microvessels to reduced oxygen tension. The results of the present study suggest that the net response of skeletal muscle resistance arteries of Sprague-Dawley rats to incremental reductions in PO2 represents an integration of NO-dependent, PGI2-dependent, and 20-HETE-dependent signaling pathways, mediated via the opening of KATP and KCa channels, with minimal evidence supporting a role for EETs in this process. In addition, the dilation of skeletal muscle resistance arteries in response to incremental hypoxia also appears to reflect primarily alterations in VSM transmembrane potential (Fig. 1C) and may not have been due to other processes [e.g., the production of substances affecting the sensitivity of the muscle contractile filaments (23)]. Furthermore, the present results clearly suggest that reactivity of these vessels to reduced oxygen tension is not wholly endothelium dependent, as ~20% of the normal vessel responses to hypoxia were retained after removal of the vascular endothelium (Fig. 2, A and B). Most interestingly, the present results suggest that each of these signaling pathways exerts its effect over a somewhat distinct range of reduced PO2 and that the relative contribution of each pathway to determining the net response of the arteries to reduced PO2 changes with the severity of hypoxia.

With mild hypoxia (15% O2), the mechanical, electrophysiological, and biochemical data obtained in the present study suggest that arterial dilation and VSM cell membrane hyperpolarization depend on the release of NO from the vascular endothelium, with minimal apparent contribution from either reduced vascular levels of 20-HETE or increased production of endothelium-derived PGI2. Additional experiments suggested that this response depends primarily on the opening of KCa channels, as vessel dilation and VSM hyperpolarization in response to mild hypoxia were unaltered after treatment of vessels with glibenclamide, but were abolished after application of iberiotoxin. With moderate reductions in PO2 (10% O2), it appears that the increased arterial dilation and the greater hyperpolarization of the VSM membrane depend less on additional NO release and are more a function of PGI2 release from the endothelium. The present results also suggest that reduced levels of 20-HETE in the vessels play a role in mediating responses of the arteries to moderate hypoxia. Data collected using the K+ channel antagonists suggest that the response of vessels to moderate hypoxia is mediated through an opening of KATP channels and an additional opening of KCa channels, because both glibenclamide and iberiotoxin attenuated vascular responses to moderate hypoxia. Finally, at the most se-
vere levels of hypoxia (5% \( \text{O}_2 \) and 0% \( \text{O}_2 \)), arterial dilation and VSM hyperpolarization appeared to depend primarily on PGI2 release from the endothelium, with minimal further contribution from either NO- or 20-HETE-dependent signaling pathways. The present results also suggest that the responses of vessels to the larger reductions in \( \text{PO}_2 \) are mediated primarily by the opening of K\(_{\text{ATP}}\) channels and are only partially dependent on the opening of K\(_{\text{Ca}}\) channels, because application of glibenclamide severely impaired these responses, whereas application of iberiotoxin resulted in a more modest attenuation.

The additional issue addressed by the present experiments pertains to which K\(^+\) channels are affected by the primary mediators of hypoxic dilation and over what range of reduced \( \text{PO}_2 \). On the basis of data presented in Fig. 8, the present results suggest that NO released during mild hypoxia activates K\(_{\text{Ca}}\) channels, causing VSM membrane hyperpolarization and vascular relaxation. This hypothesis is supported by observations that application of L-NAME had no effect on vessels treated with iberiotoxin, but abolished vascular responses to mild hypoxia in arteries treated with glibenclamide. It is also likely that the reduction in vascular 20-HETE levels during moderate hypoxia also causes the opening of K\(_{\text{Ca}}\) channels, leading to VSM hyperpolarization and vascular relaxation. The latter conclusion is supported by a previous study demonstrating that 20-HETE inhibits the opening of K\(_{\text{Ca}}\) channels in renal microvessels and that decreasing 20-HETE concentration removes this inhibition (25).

As with NO, this appears to be a reasonable hypothesis, given that treatment of vessels with 6(Z),15(Z)-20-HETE impaired vasodilator responses to reduced \( \text{PO}_2 \) in vessels treated with glibenclamide, but had no effect on vessels treated with iberiotoxin, where the channels that are presumably inhibited by 20-HETE were already blocked. Furthermore, in agreement with previous studies (5, 17), the PGI2 produced during moderate to severe hypoxia appears to exert its effects via K\(_{\text{ATP}}\) channels, because application of indomethacin to vessels treated with glibenclamide had no additional effect on vessel responses to incremental hypoxia, but application of the COX inhibitor indomethacin to arteries

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**Fig. 7.** Changes in the release of nitric oxide (A) and 6-keto-PGF\(_{1\alpha}\) (the stable breakdown product of PGI2; B) by pooled gracilis arteries and vessel 20-HETE content (C) in response to incremental reductions in oxygen content of the bathing medium. Data are presented as means \( \pm \) SE. Data describing metabolite production by gracilis arteries under normoxic conditions (21% \( \text{O}_2 \)) are presented in Table 1. Production levels of all metabolites are normalized to the wet weight of the pooled arteries in the incubation medium. *\( P < 0.05 \) vs. metabolite production levels under 21% \( \text{O}_2 \).
treated with iberiotoxin abolished the dilation of gracilis arteries in response to reduced \( \text{PO}_2 \). Finally, the biochemical data describing the production of these three mediators of hypoxic dilation provide additional support for these hypotheses, in that the \( \text{PO}_2 \) range over which the production of the individual metabolites occurs appears to be highly correlated with mechanical and electrophysiological responses of these vessels to incremental hypoxia. It is important to note that experiments aimed at determining mechanical and electrophysiological responses of isolated vessels in response to graded hypoxia employed cannulated, pressurized resistance arteries, whereas experiments determining metabolite production with incremental hypoxia used pooled (i.e., nonpressurized) vessels. Although these experimental conditions are not directly comparable and the effects of intraluminal pressure on metabolite production in this model are not well defined, to our knowledge, these represent the first systematic studies of the production and/or release of these metabolites with progressive reductions in \( \text{PO}_2 \).

One intriguing observation of the present study was that pharmacological inhibition of the vasoconstrictor metabolite 20-HETE did not alter resting arterial tone. Although puzzling, our observation is consistent with results of previous studies of in situ skeletal muscle arterioles (11, 16), in vitro skeletal muscle arterioles (7, 14), and in vitro renal arteries and arterioles (2). Furthermore, vessel dilation after CP450 4A enzyme inhibition has only been identified after endothelium removal in isolated renal (2) and skeletal muscle arteries (7). Hypotheses of possible mechanisms explaining how alleviation of a constrictor influence (20-HETE) causes dilation of endothelium-denuded vessels only recently have been forwarded (2). In that study, the authors hypothesized that CP450 4A enzyme inhibitors may not alter diameter of intact arterioles as they block formation of both a vasoconstrictor in VSM cells and a vasodilator in the endothelium, thus generating a measurable effect in endothelium-denuded vessels only. A second hypothesis forwarded in that study suggested that basal NO release from the endothelium inhibits the formation of 20-HETE in VSM cells, limiting 20-HETE concentration. Endothelium removal eliminates this effect and allows 20-HETE levels to rise in VSM, thus CP450 enzyme inhibitors would block a higher level of 20-HETE production in endothelium-denuded vessels, causing the observed dilator response (2). These hypotheses warrant future investigation.

The results of the present study may provide insight into the existing divergence of opinion regarding the mediators of hypoxic dilation in skeletal muscle microvessels. Previous studies from our laboratory and others have indicated that the reactivity of skeletal muscle microvessels in response to alterations in \( \text{PO}_2 \) may depend on NO release (14, 22), PG\( \text{I}_2 \) release (5, 7, 15, 17, 19, 20), or EET release (6) from the endothelium and could also depend, in part, on a reduction in 20-HETE levels within VSM cells (7, 11, 16). Possible explanations for the disparity in these results may reflect issues of differences in the specific tissue used [gracilis muscle (5–8) vs. spinotrapezius muscle (22) or cremaster muscle (11, 16, 19, 20); longitudinal position within microvascular networks [resistance arteries (5–8) vs. large arterioles (14, 19, 20) vs. distal arterioles (11, 16, 22)]; the use of in situ preparations (11, 16, 22) vs. in vitro preparations (5–8, 14, 17, 19, 20); or animal species and strain differences [Sprague-Dawley (7, 8, 11, 15, 19, 20, 22) vs. Dahl rats (6) or rats vs. hamsters (16)].

In conclusion, when integrated, the data from the present study, which combine measurements of mi-
crovessel diameter, VSM transmembrane potential, and biochemical analyses under a series of physiologically and pharmacologically imposed conditions, may begin to provide a more informative framework for understanding the response of skeletal muscle resistance arteries to hypoxia (from 21% O₂ control). The relative contribution of the different chemical mediators and potassium channels to hypoxic dilation is estimated by averaging their individual contribution to the mechanical responses (% of normal dilation to reduced P₀₂) and electrophysiological responses (% of normal VSM hyperpolarization to a reduced P₀₂, which were highly predictive of the mechanical responses) of the vessel to incremental reductions in oxygen tension.

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Fig. 9. A schematic representation of the results of the present study. Data are summarized to indicate the relative contribution of the 3 predominant metabolites (nitric oxide, prostacyclin, or 20-HETE) and the 2 predominant potassium channels (KᵥCa and KᵥATP) in mediating the dilation of skeletal muscle resistance arteries to incremental hypoxia (from 21% O₂ control). The relative contribution of the different chemical mediators and potassium channels to hypoxic dilation is estimated by averaging their individual contribution to the mechanical responses (% of normal dilation to reduced P₀₂) and electrophysiological responses (% of normal VSM hyperpolarization to a reduced P₀₂, which were highly predictive of the mechanical responses) of the vessel to incremental reductions in oxygen tension.

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