Chronic hypoxia and VEGF differentially modulate abundance and organization of myosin heavy chain isoforms in fetal and adult ovine arteries

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Hubbell MC, Semotiuik AJ, Thorpe RB, Adeoye OO, Butler SM, Williams JM, Khorram O, Pearce WJ. Chronic hypoxia and VEGF differentially modulate abundance and organization of myosin heavy chain isoforms in fetal and adult ovine arteries. Am J Physiol Cell Physiol 303:C1090–C1103, 2012. First published September 19, 2012; doi:10.1152/ajpcell.00408.2011.—Chronic hypoxia increases vascular endothelial growth factor (VEGF) and thereby promotes angiogenesis. The present study explores the hypothesis that hypoxic increases in VEGF also remodel artery wall structure and contractility through phenotypic transformation of smooth muscle. Pregnant and nonpregnant ewes were maintained at sea level (normoxia) or 3,820 m (hypoxia) for the final 110 days of gestation. Common carotid arteries harvested from term fetal lambs and nonpregnant adults were denuded of endothelium and studied in vitro. Stretch-dependent contractile stresses were 32 and 77% of normoxic values in hypoxic fetal and adult arteries. Hypoxic hypocontractility was coupled with increased abundance of nonmuscle myosin heavy chain (NM-MHC) in fetal (+37%) and adult (+119%) arteries. Conversely, hypoxia decreased smooth muscle MHC (SM-MHC) abundance by 40% in fetal arteries but increased it 123% in adult arteries. Hypoxia decreased colocalization of NM-MHC with smooth muscle α-actin (SM-α-A) in fetal arteries and decreased colocalization of SM-MHC with SM-α-A in adult arteries. Organ culture with physiological concentrations (3 ng/ml) of VEGF-A165 similarly depressed stretch-dependent stresses to 37 and 49% of control fetal and adult values. The VEGF receptor antagonist vatalanib ablated VEGF’s effects in adult but not fetal arteries, suggesting age-dependent VEGF receptor signaling. VEGF replicated hypoxic decreases in colocalization of NM-MHC with SM-α-A in fetal arteries and decreases in colocalization of SM-MHC with SM-α-A in adult arteries. These results suggest that hypoxic increases in VEGF not only promote angiogenesis but may also help mediate hypoxic arterial remodeling through age-dependent changes in smooth muscle phenotype and contractility.

Remodeling alters the shape and size of arterial smooth muscle cells (18, 59) with corresponding changes in contractility and vascular reactivity (31, 33, 38). These changes in structure and function are presumably coupled with transformations of smooth muscle phenotype, which can be identified by characteristic changes in key contractile proteins (31, 49). In turn, changing the abundance of smooth muscle α-actin (SM-α-A; Refs. 44, 45), together with other contractile proteins, such as myosin heavy chain (MHC) isoforms (49, 50), reliably and dynamically reveals corresponding changes in smooth muscle phenotype during both physiological (31) and pathophysiological (23, 59, 66) vascular remodeling.

The exact mechanisms that drive vascular remodeling and phenotypic transformation of smooth muscle remain poorly understood but appear to include the actions of a broad variety of growth factors including fibroblast growth factor (48), platelet-derived growth factor (21), transforming growth factor-β (6), and vascular endothelial growth factor (28, 44). Some vasotropic factors originate from local metabolically active parenchymal cells and act primarily at the serosal surface of blood vessels (1), whereas others such as endothelin and nitric oxide (10, 15), emanate from the vascular endothelium and act more prominently at the luminal surface. Others are released into both the interstitium and the bloodstream and thereby coordinate widely distributed effects on vascular structure and function. An excellent example in this latter category is vascular endothelial growth factor (VEGF). Long recognized as a key mediator of angiogenesis (16), VEGF also can exert trophic effects on nonendothelial cell types including pericytes (61), central nervous system neurons (32), astrocytes (22), Schwann cells (58), sympathetic neurons (39), skeletal muscle (26, 43), and smooth muscle (11, 26, 43). Equally important, the synthesis and release of VEGF are strongly stimulated by hypoxia via multiple pathways (20, 62). Together, these characteristics give VEGF the potential to broadly influence vascular remodeling and phenotypic transformation of smooth muscle, particularly in response to chronic hypoxia.

The present study explores the hypothesis that VEGF may contribute to age-dependent hypoxic remodeling of artery structure and function through changes in contractile protein abundance and organization secondary to changes in smooth muscle phenotype. In the context of this study, artery “structure” was assessed through measurements of medial layer thickness, unstressed diameter, and the transmural distribution and intracellular organization of smooth muscle contractile proteins. In turn, intracellular organization and smooth muscle phenotype were defined by the patterns of colocalization of nonmuscle and smooth muscle myosin heavy chain (NM-MHC...
and SM-MHC) with SM-αA (49, 50). The experimental design focused first on the effects of chronic hypoxia on artery structure, contractile protein organization, and function using established methods for immunoblotting, confocal microscopy, and measurements of in vitro contractility (11, 14). The same endpoints also helped define the structural and functional effects of organ culture with VEGF in a second tier of experiments. These experiments employed VEGF at 3 ng/ml, a low physiologically relevant concentration (19, 64) that minimized nonspecific activation of non-VEGF receptors (4). To minimize influences attributable to the release of parenchymal metabolites, we performed all measurements in carotid arteries. Comparisons between arteries harvested from sheep maintained at sea level and those maintained at high altitude (3,820 m) for 110 days served to define the effects of chronic hypoxia, as previously described (47). Because vascular remodeling manifests very differently in mature and immature arteries (12), the experimental design also included comparisons between arteries harvested from nonpregnant adult sheep and term fetal lambs. Together, these approaches enabled a unique perspective of the role of VEGF in age-dependent hypoxic vascular remodeling.

MATERIALS AND METHODS

All procedures used in these studies were approved by the Animal Research Committee of Loma Linda University, adhered to the policies and practices set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and have been previously described in detail (11, 67).

Tissue harvest and preparation. Common carotid arteries were harvested using sterile techniques from fetal (139–142 days gestation) and young nonpregnant adult sheep (18–24 mo old) that had been maintained at either sea level (normoxic) or at 3,820 m for 110 days and young nonpregnant adult sheep and term fetal lambs. Together, these approaches enabled a unique perspective of the role of VEGF in age-dependent hypoxic vascular remodeling.

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Arterial sections were exposed to primary antibodies overnight with polyclonal rabbit anti-human NM-MHC (CoVance, Princeton, NJ; PPR-445P) at 1:500 or polyclonal rabbit anti-bovine SM-MHC (Abcam, Cambridge, MA; ab53219) at 1:500. The following day, slides were washed for two 10-min cycles in PBS and incubated using the appropriate secondary antibody with DyLight 488 conjugated (Pierce Chemical, Rockford, IL; no. 35502) for 2 h at room temperature. The secondary antibody was a goat anti-rabbit-488 (Thermo Scientific, Rockford, IL; no. 35552 lot L1150311). To minimize photobleaching of the fluorescent dyes, slides were stored in the dark. Following two 10-min cycles in PBS, tissue slides were coverslipped using SlowFade Gold anti-fade reagent with DAPI (Invitrogen, Carlsbad, CA; S36939) and then stored until imaged. All images were captured using a Zeiss AXIO Imagem A1 fluorescence microscope and Spot software (Diagnostic Instruments, Ver 4.6.4.5).

**Transmural morphometry.** As previously described in detail (11), artery sections processed for fluorescent immunohistochemistry were analyzed using standard morphometric procedures to quantify the distribution of contractile proteins across the artery wall. For this analysis, six individual line-intensity scans were recorded along radial lines extending from the basal lamina to the adventitial-medial junction using Image Pro Plus (Media Cybernetics, Version 6.0). The raw line scans mapped fluorescent intensity vs. distance from the lumen and were equally distributed at 60° increments around the lumen. Distance measurements were normalized to medial thickness with a value of “0” assigned to the region just inside the basal lamina and a value of “100” assigned to the region just inside the adventitial-medial junction. Following published procedures (11), separately determined calibration curves were used to convert fluorescent intensities into relative fluorophore concentrations and thereby linearize estimates of signal intensities and corresponding regional antigen concentration. These estimates were averaged as a function of relative distance from the lumen for all line scans from the same section. The “inner,” “middle,” and “outer” regions were averaged across values from 5–20, 45–60, and 80–95% of the normalized distance from the lumen, respectively. The innermost 5% and outermost 5% of the medial layer were excluded to avoid autofluorescent contamination from the basal elastic lamina and adventitial elastin and collagen, respectively. These lines determined the relative distribution of each marker across the artery wall. The absolute abundance of each marker in the whole artery wall was determined by semiquantitative Western blot. For each marker, the area beneath the distance-concentration curves was normalized to 100% and then multiplied by the Western blot values to calculate relative local abundance values that could be compared between regions and experimental groups.

**Confocal microscopy.** Matched artery segments were collected, prepared and cut into 5 μm sections, deparaffinized, sectioned, and immunostained as described in **Transmural morphometry** with the exception that all sections were stained with primary monoclonal mouse anti-sheep antibodies against SM-αA (Sigma-Aldrich; A5228, 1:200) and a secondary primary antibody against one of the MHC isoforms. Because in arterial smooth muscle the α isoform of actin is the main isoform involved in smooth muscle contraction (45), all colocalization of MHC was quantified relative to the abundance of α-actin. Because only α-actin is involved in smooth muscle contraction, changes in the other actin isoforms that can also be expressed in smooth muscle (45) should have little if any influence on smooth muscle contractility. The antibodies used to detect the MHC isoforms were as described in **Fluorescent immunohistochemistry.** Following incubation with the primary antibodies, sections were washed in PBS and equilibrated in darkness for 2 h at room temperature with two secondary antibodies labeled with DyLight 488 to detect SM-αA and DyLight-649 to detect MHC, as indicated in **Fluorescent immunohistochemistry.** Following secondary staining, sections were coverslipped using SlowFade Gold Antifade Reagent (S36936; Invitrogen) and then examined with an Olympus FV1000 confocal microscope at an optical section thickness of 0.7 μm, a lateral resolution of 200 nm, and a numerical aperture of 1.4.

Antigen colocalization in confocal images was analyzed using FlouView software (version 2.1c), which provided multiple indices of colocalization including the Manders Colocalization Index 1 (actin signal in the denominator) that quantifies the portion of pixels that fluoresce for both MHC and SM-αA relative to the total number of pixels positive for SM-αA. Positive pixels were defined as having a fluorescent intensity ≥5% of the maximum fluorescence intensity in the section. To eliminate cells with a small but significant above-threshold abundance of SM-αA characteristic of incompletely differentiated smooth muscle cells and some non-smooth muscle cell types (55), a second index of colocalization was developed. This second index, which we termed percent upper right (%UR), was calculated to select for differentiated contractile smooth muscle as identified by high SM-αA abundance levels. This index counted only pixels that fluoresced at or above the mean intensity threshold for SM-αA, which in essence was equivalent to recalculating the Colocalization Index 1 with an SM-αA threshold of 50% of maximum intensity. This method of analysis was derived from a flow-cytometry quadrant analysis and thus was referred to as the percentage in the upper right quadrant of the scatterplot of SM-αA intensities against MHC intensities. From a general perspective, Colocalization Index 1 and %UR were both proportional to the fraction of actin-positive pixels that were positive for MHC. Interestingly, the results obtained for both Colocalization Index 1 and %UR varied somewhat in absolute values but were qualitatively similar.

**Immunoblotting.** Artery segments were homogenized via glass on glass in 8 M urea, 500 mM NaCl, 23 mM glycin, 20 mM Tris, 10 mM EGTA, and 10% glycerol at pH 8.6 with the addition of a protease inhibitor cocktail at 5 μl per ml of buffer (Sigma-Aldrich; no. M1745) that included the following (final concentrations): 52 mM AEBSF, 2 mM bestatin, 1 mM leupeptin, 750 μM pepstatin A, 700 μM E-64, and 40 μM aprotinin. Centrifugation of the homogenate at 5,000 g for 20 min yielded a supernatant in which protein concentration was determined using the Bio-Rad Bradford assay. Optimal dilutions of total soluble protein in the extracted supernatant were loaded after which the proteins were separated by SDS-PAGE alongside pooled reference standards prepared from adult ovine common carotid arterial tissue to calibrate sample abundances on each gel. Separated proteins were transferred to nitrocellulose at 350 mA for 90 min in Towbin’s buffer (192 mM glycine, 25 mM Tris, and 10% methanol for MHC and 20% methanol for SM-αA) with β-mercaptoethanol added to the upper buffer reservoir. Membranes were blocked with 5% milk in Tris-buffered saline at pH 7.5 (M-TBS) for 1 h at room temperature using continuous shaking. All subsequent washes and incubations were done in M-TBS with 0.1% Tween-20. Membranes were incubated with the same primary antibodies listed above at the following concentrations: anti-SM-αA, 1:3,000; anti-NM-MHC, 1:1,000; and anti-SM-MHC, 1:20,000 for 3 h. For visualization, membranes were incubated for 90 min with a secondary antibody conjugated to DyLight 800 (Pierce Chemical; no. 46422) and imaged on a LI-COR Bioscience’s Odyssey system. All protein abundances were expressed as the equivalent mass of standard relative to the mass of protein loaded in each lane.

**Data analysis and statistics.** Contractile stresses were calculated as ratios of force per cross-sectional area, where force was calculated as contractile tension in grams times the acceleration due to gravity. Cross-sectional area (wall thickness × segment length) was corrected for changes with stretch as described previously (30, 46). Stress values were normalized within each artery segment by calculating the percentage of the maximum force produced by the artery exerted at each stretch ratio. Percent maximum values were converted back into absolute units of stress by multiplying by the average maximum stress calculated across all segments within the same experimental group.

Immunofluorescence intensity values were recorded as a function of relative radial distance from the lumen to the adventitia and then
normalized within each segment to yield an area beneath the intensity-distance curve of unity, as previously described in detail (11). Fluorescence intensity values were calibrated against relative marker abundance measured with Western blots, which in turn were calibrated for each marker against a standard curve pooled from adult common carotids. For confocal colocalization, all values were calculated among SM-αA-positive pixels and determined the fractions of those pixels also positive for each of the MHC isoforms. The total colocalization values were obtained by adding the individual Colocalization Index values for each of the MHC isoforms, and the group error was calculated as a pooled variance. All statistical comparisons were done using Behrens-Fisher comparison at the $P < 0.05$ level with each animal contributing equally to each treatment group.

RESULTS

A total of 174 endothelium-denuded carotid artery segments were harvested from 8 normoxic fetuses (FN), 7 hypoxic fetuses (FH), 11 normoxic adults (AN), and 8 hypoxic adults (AH). When duplicate segments from a single animal were used in the same protocol, the resulting values were averaged and the resulting average was treated as a single observation. Throughout the text, “n” denotes the number of animals used in each experiment, not the number of segments. Statistical significance was defined at $P < 0.05$ for all assays, with all values given as means ± SE.

Chronic hypoxia altered age-dependent contractile function of large arteries. Normoxic values of medial wall thicknesses and unstressed diameters were significantly less in fetal than in adult arteries, and these values were not significantly different than age-matched hypoxic values (Table 1). Maximum stretch-dependent stresses were significantly greater in fetal than in adult arteries and were significantly less in hypoxic than in normoxic arteries from both age groups (Fig. 1). Conversely, maximum K$^+$-induced stresses were significantly greater in adult than in fetal arteries and were decreased only modestly by hypoxia in both age groups.

### Chronic hypoxia altered age-dependent MHC abundance and distribution

Relative abundances of the embryonic isoform of MHC (NM-MHC) were significantly greater in fetal than adult arteries and were similarly increased by hypoxia in both age groups. Normoxic fetal values averaged 1.35% of normoxic adult values, and hypoxic fetal values averaged 850% of hypoxic adult values (Fig. 2). In hypoxic arteries, NM-MHC abundance values averaged 137 and 216% of normoxic values in fetal and adult arteries, respectively. In normoxic fetal arteries, the regional abundance of NM-MHC (in μg/μg standard) was significantly greater in the inner medial region (7.87 ± 0.67) than in either the middle medial (4.82 ± 0.33) or outer medial (4.75 ± 0.42) regions (Fig. 2). A similar pattern was observed across the inner medial (9.90 ± 1.11), middle medial (6.94 ± 0.74), and outer medial (7.03 ± 0.76) regions of hypoxic fetal arteries. In adult arteries, NM-MHC abundance values did not vary significantly among regions in either normoxic or hypoxic arteries.

For SM-MHC, relative abundance was significantly less in fetal than in adult arteries; fetal values averaged only 62% of adult values in normoxic arteries and only 17% of adult values in hypoxic arteries. Hypoxia significantly decreased SM-MHC abundance to 60% of normoxic values in fetal arteries but increased it to 223% of normoxic values in adult arteries. Distribution was homogenous among all artery wall regions in all four treatment age groups (Fig. 2).

### Hypoxia altered colocalization of MHC isoforms and SM-αA

Consistent with the abundance results, colocalization of NM-MHC with SM-αA in normoxic fetal arteries averaged 1.82% of colocalization in normoxic adult arteries (Fig. 3) and in hypoxic fetal arteries averaged 880% of colocalization in hypoxic adult arteries; maturation markedly decreased NM-MHC colocalization. Hypoxia depressed NM-MHC colocalization to 52% of normoxic values in fetal arteries ($P < 0.05$) but was without effect in adult arteries (Fig. 3).

### Table 1. Effects of hypoxia and VEGF on artery structure and function

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Medial Thickness, μm</th>
<th>Unstressed Diameter, μm</th>
<th>Stretch-Dependent Maximum Stress, dyn/cm²</th>
<th>K$^+$-Induced Maximum Stress, dyn/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fetal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normoxic</td>
<td>203 ± 18$^A$</td>
<td>2,900 ± 290$^A$</td>
<td>19,730 ± 6,460$^{A,H}$</td>
<td>6,370 ± 1,890$^A$</td>
</tr>
<tr>
<td>Hypoxic</td>
<td>221 ± 21$^A$</td>
<td>3,230 ± 250$^A$</td>
<td>6,280 ± 1,220$^{A,H}$</td>
<td>5,240 ± 1,170$^A$</td>
</tr>
<tr>
<td><strong>Adult</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normoxic</td>
<td>543 ± 25$^A$</td>
<td>4,020 ± 310$^A$</td>
<td>8,600 ± 1,730$^{A,H}$</td>
<td>31,220 ± 3,530$^A$</td>
</tr>
<tr>
<td>Hypoxic</td>
<td>591 ± 30$^A$</td>
<td>3,760 ± 220$^A$</td>
<td>6,660 ± 1,730$^{A,H}$</td>
<td>28,640 ± 4,460$^A$</td>
</tr>
<tr>
<td><strong>Fetal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>207 ± 10$^A$</td>
<td>3,149 ± 210$^A$</td>
<td>17,510 ± 4,995$^{A,V}$</td>
<td>6,037 ± 636$^{A,1}$</td>
</tr>
<tr>
<td>VEGF</td>
<td>211 ± 14$^A$</td>
<td>3,339 ± 296$^A$</td>
<td>6,541 ± 2,506$^{A,V}$</td>
<td>7,264 ± 887$^A$</td>
</tr>
<tr>
<td>VEGF + Vat</td>
<td>231 ± 10$^A$</td>
<td>2,498 ± 91$^A$</td>
<td>7,279 ± 1285$^A$</td>
<td>9,995 ± 1,945$^{A,1}$</td>
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<tr>
<td><strong>Adult</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>469 ± 21$^A$</td>
<td>4,742 ± 204$A$</td>
<td>30,756 ± 7,685$^{A,V}$</td>
<td>8,167 ± 1,773$^V$</td>
</tr>
<tr>
<td>VEGF</td>
<td>501 ± 26$A$</td>
<td>4,645 ± 270$^A$</td>
<td>15,192 ± 3,737$^{A,V,I}$</td>
<td>4,479 ± 1,260$^V$</td>
</tr>
<tr>
<td>VEGF + Vat</td>
<td>478 ± 22$A$</td>
<td>4,754 ± 206$^A$</td>
<td>25,475 ± 7,876$^{A,V,I}$</td>
<td>6,990 ± 1,197$^V$</td>
</tr>
</tbody>
</table>

Indicated are means ± SE for arteries from 8 normoxic fetuses, 4 hypoxic fetuses, 11 normoxic adults, and 8 hypoxic adults. Medial thicknesses were measured in unfixed specimens using phase contrast light microscopy. Unstressed diameters were measured in arteries mounted in vitro for contractility. Stretch-dependent maximum (myogenic) stresses and maximum active stresses induced by exposure to isotonic Krebs buffer containing 122 mM K$^+$ were averaged across arteries independent of the strain at which the maximum was observed. Note that the average of the maximum potassium-induced stresses (above) was greater than the maximum of the average potassium-induced stresses plotted against stretch-ratio in Fig. 4. This difference implies that the stretch ratios at which maximum contractility was observed varied markedly among different segments. All normoxic and hypoxic values were obtained in freshly dissected arteries, whereas control, VEGF, and VEGF + vatalanib (Vat) values were obtained in arteries organ cultured 48 h. Control, VEGF, and VEGF + Vat means were calculated from 6 normoxic fetal lambs and 11 normoxic adult sheep. Superscripts A, H, V, and I denote significant differences ($P < 0.05$) due to age, hypoxia, VEGF treatment, and VEGF receptor inhibitor treatment, respectively.
Fig. 1. Chronic hypoxia decreases myogenic contractility. Endothelium-denuded common carotid arteries harvested from fetal and adult sheep maintained at 3,820 m for 110 days (hypoxic) or at sea level (normoxic) were used to determine stretch-induced myogenic stresses at different strains applied in increments of unstressed diameter (D/D₀). Myogenic stresses were calculated as the spontaneous stresses measured under resting conditions at each strain ratio (D/D₀) minus the passive stresses measured after freezing in liquid N₂ at the same strain ratio. Active potassium-induced stresses were induced by exposure to isotonic Krebs buffer containing 122 mM K⁺. Potassium-induced stresses were calculated as the maximum stresses measured during exposure to high potassium at each strain ratio (D/D₀) minus the spontaneous stresses measured under resting conditions at the same strain ratio. Note that spontaneous myogenic stresses were greater in fetal than adult arteries (top), whereas active potassium-induced stresses were less in fetal than adult arteries (bottom). Error bars indicate SE for arteries from 8 normoxic adults, and 8 hypoxic adults. *P < 0.05, significant differences between normoxic and hypoxic values.

Although SM-MHC abundance was significantly less in fetal than adult arteries (Fig. 2), colocalization of SM-MHC with SM-αA was greater in fetal than adult arteries. SM-MHC and SM-αA colocalization also was not affected by chronic hypoxia in fetal arteries but was decreased by hypoxia in adult arteries. Colocalization values in normoxic fetal arteries were 220% of values in normoxic adult arteries, and in hypoxic fetal arteries colocalization averaged 390% of hypoxic adult arteries (Fig. 3).

Effects of VEGF and VEGF receptor antagonism on age-dependent contractility. To assess the potential role of VEGF in hypoxic vascular remodeling, the effects of VEGF on vascular contractility were determined in a separate series of arteries cultured in the presence and absence of the VEGF receptor antagonist vatalanib (Fig. 4) (35). Medial wall thicknesses and unstressed diameters were significantly less in fetal than adult arteries in each treatment group and did not vary with treatment in either age group (Table 1).

Maximum stretch-dependent stresses averaged less in fetal than adult arteries in all three treatment groups (Table 1). In fetal arteries, maximum stretch-dependent stresses in VEGF-treated and VEGF + vatalanib-treated arteries were only 37 and 42% of control values, respectively (P < 0.05) and did not differ from one another. In adult arteries, average stresses in the VEGF and VEGF + vatalanib groups were 55% (P < 0.05) and 86% (NS) of control values; in adult arteries vatalanib prevented the effects of VEGF such that stretch-dependent stresses in the VEGF + vatalanib group were significantly greater than in the VEGF group and were similar to the control group.

Effects of VEGF on age-dependent MHC abundance and distribution. To explore the effects of serum starvation alone on MHC isoform expression, abundances in normoxic fresh, uncultured arteries (Fig. 2) were compared with corresponding values in serum-starved control arteries (Fig. 5). These comparisons revealed that in contrast to the marked effects of serum starvation on the abundance of other contractile proteins such as myosin light chain kinase (11), serum starvation had only modest effects on the abundance of MHC isoforms in both fetal and adult arteries. To control for these changes, all effects of VEGF were determined by comparisons between “starved” and “VEGF-treated” segments. With this approach, all arteries underwent a standardized organ culture regimen with treatments between groups varying only by the presence or absence of VEGF and/or vatalanib.

The relative abundance of NM-MHC in control and VEGF-treated fetal arteries was 1,960 and 1,740% of abundance in corresponding adult arteries (Fig. 5). VEGF treatment increased NM-MHC abundance in fetal arteries, but this effect was significant only in the inner medial region. VEGF had no significant effect on NM-MHC abundance in adult arteries. In control fetal arteries, the local abundance (in µg/µg standard) of NM-MHC was significantly greater in the inner medial region (5.35 ± 0.78) than in the middle medial region (3.88 ± 0.55), which in turn was greater than in the outer medial (2.54 ± 0.37) region (Fig. 5). A similar pattern was observed across the inner medial (6.64 ± 0.53), middle medial (4.43 ±
0.36), and outer medial (2.99 ± 0.22) regions of VEGF-treated fetal arteries. In adult arteries, NM-MHC abundances did not vary significantly among regions in either control or VEGF-treated arteries.

For SM-MHC, the relative abundances were significantly less in control fetal than in control adult arteries. VEGF treatment did not significantly influence the abundances of SM-MHC in either fetal or adult arteries, and these abundances remained significantly less in VEGF-treated fetal than in VEGF-treated adult arteries (Fig. 5). Regional abundances of SM-MHC did not vary significantly across the different transmural regions in control or VEGF-treated arteries from either age group.

**VEGF altered colocalization of MHC with SM-αA.** Colocalization of NM-MHC with SM-αA in control fetal arteries averaged 710% of control adult arteries (Fig. 6). VEGF treatment reduced NM-MHC colocalization to only 32 and 36% of control values in fetal and adult arteries, respectively. After VEGF treatment, NM-MHC colocalization remained markedly greater in fetal than adult arteries.

![Fig. 2. Chronic hypoxia alters myosin heavy chain (MHC) abundance and distribution.](image-url)
that VEGF may contribute to age-dependent hypoxic remodeling of artery structure and function through changes in contractile protein abundance and organization secondary to changes in smooth muscle phenotype.

Hypoxic acclimatization altered artery structure and function. The effects of hypoxic acclimatization on artery wall thicknesses vary with artery type, size, and age (38, 60, 68) and can differentially affect the endothelial, medial, and adventitial layers (59, 67). To better understand the functional consequences of hypoxic changes in artery structure, the present study focused on the medial layer that contains the smooth muscle cells responsible for contractile force. In both adult and fetal ovine carotid arteries, hypoxic acclimatization had no significant effect on medial thicknesses, which suggests that any changes in artery contractility induced by hypoxia must have been due to changes in either the abundances or organization of contractile proteins within the arterial smooth muscle.

In many vascular studies, contractile responses are measured only in units of grams or as percentages of responses to a standard contractant such as high potassium at a single segment length or passive tension. These approaches can provide excellent internal normalization but do not account for differences in wall thickness, contractile protein content, stiffness, or optimum length, which can be significant when comparing contractility across different chronic treatment groups. To avoid these limitations, our experimental approach included complete stress-strain determinations for each segment in which contractile responses were quantified as changes in active stresses measured in units of force per medial cross-section of artery wall and as percentages of control responses. These approaches can provide excellent internal normalization but do not account for differences in wall thickness, contractile protein content, stiffness, or optimum length, which can be significant when comparing contractility across different chronic treatment groups. To avoid these limitations, our experimental approach included complete stress-strain determinations for each segment in which contractile responses were quantified as changes in active stresses measured in units of force per medial cross-section of artery wall and as percentages of control responses.
This disconnect between the effects of hypoxia on K+-induced contractions was greater in adult than fetal arteries (11) and were not dramatically attenuated by hypoxia (38). In contrast, stretch-dependent (myogenic) contractions were of greater magnitude in fetal than adult arteries (11) and were not dramatically attenuated by hypoxia (38). In contrast, stretch-dependent (myogenic) contractions were of greater magnitude in fetal than adult arteries but were similarly and significantly attenuated by hypoxic acclimatization in both age groups (Table 1). This disconnect between the effects of hypoxia on K+-induced and stretch-dependent (myogenic) contractions demonstrates that hypoxic acclimatization can differentially influence select components of the contractile apparatus and may preferentially affect mechanisms coupling stretch to contraction. Candidate mechanisms potentially involved in this selective effect of hypoxia include calcium channels involved in mechanotransduction (27) and contractile proteins involved in myogenic regulation of myofilament calcium sensitivity (14, 52). Interestingly, many of these mechanisms are strongly influenced by phenotypic transformation of smooth muscle (14, 29, 65).

Hyoxia acclimatization altered MHC abundance and organization. To explore the hypothesis that hypoxic changes in artery structure and contractility may be secondary to changes in smooth muscle phenotype, our experiments focused on changes in the abundance of MHC isoforms that are closely associated with both contractile capacity and smooth muscle phenotype (23, 24, 49). Because smooth muscle morphology and phenotype are highly heterogeneous in the artery wall (18, 60), our measurements of MHC abundance combined homogenate immunoblots calibrated against known standards, with radial line scans of fluorescent intensity in immunostained coronal sections to estimate regional protein abundance across the artery wall, as previously described (11). This approach revealed that the embryonic isoforms of MHC (NM-MHC), which are prevalent in functionally immature and proliferative smooth muscle cells (17, 44, 49), were more abundant in fetal than adult arteries, were more abundant in hypoxic than normoxic arteries in both age groups, and exhibited significant regional variability with highest abundance near the lumen in fetal arteries (Fig. 2). Together, these NM-MHC results support the hypothesis that hypoxia increases phenotypic transformation of arterial smooth muscle. The regionality of this effect in fetal arteries further suggests that vasotrophic factors released from the vascular endothelium, which was present and presumably functional in both normoxic and hypoxic fetal arteries before harvest, may influence intramural distribution and abundance of NM-MHC. Another possibility is that hypoxia stimulated changes in the expression of growth factor receptors on smooth muscle cells and thereby enhanced regional heterogeneity in reactivity to vasotrophic factors with subsequent regional changes in contractile protein abundance. Although this lamellar heterogeneity could be a simple consequence of growth factor gradients with little functional significance, it seems more likely that this heterogeneity is an important feature of arterial homeostasis that dynamically distributes roles for contractile function and secretory function throughout the smooth muscle cells of the artery wall.

Whereas the NM-MHC isoforms of MHC are coded by at least three different genes, the other primary isoforms of smooth muscle myosin (SM-MHC) arise from a single gene (17). More importantly, the contractile characteristics of the NM-MHC and SM-MHC isoforms of MHC differ significantly from one another. Correspondingly, NM-MHC appears more commonly in functionally immature smooth muscle, and SM-MHC is more common in fully differentiated contractile smooth muscle (7, 17, 44). Consistent with this pattern, the abundance of the NM-MHC isoform was significantly greater in fetal than adult arteries. In addition, fetal and adult NM-MHC abundances were significantly increased by hypoxic starvation conditions (control), with 3 ng/ml VEGF (VEGF), or with 3 ng/ml VEGF + 240 nM vatalanib (VEGF + vatalanib). With this approach, all arteries underwent a standardized organ culture regimen with treatments between groups varying only by the presence or absence of VEGF and/or vatalanib. Following organ culture, stress-strain relations were determined; at each level of strain examined both stretch dependent myogenic stress (top), and the active response to depolarization with 122 mM potassium (bottom) were determined. VEGF decreased stretch-dependent stresses in both fetal and adult arteries, but decreased potassium-induced stresses only in adult arteries. Vatalanib prevented the effects of VEGF on myogenic and active stresses only in adult arteries. *P < 0.05, significant differences between control and VEGF treatment (Behrens Fisher). Error bars indicate SE for the n values given in Table 1.
acclimatization (Fig. 2), suggesting that hypoxia promoted either proliferation of noncontractile smooth muscle or stimulated dedifferentiation of fully differentiated contractile smooth muscle. Both of these mechanisms would help explain the observed hypoxic attenuation of contractility (Fig. 1).

Consistent with the relevant literature (31, 44, 49), the overall abundance of the SM-MHC isoform was markedly less in fetal than in adult arteries, which helps explain the greater peak contractile responses to potassium in adult compared with fetal arteries (Fig. 1, bottom). Relative to normoxic abundances of SM-MHC, chronic hypoxia significantly increased SM-MHC in adult arteries but reduced SM-MHC in fetal arteries (Fig. 2). As might be expected, the hypoxic decrease in SM-MHC abundance in fetal arteries was associated with decreased contractility (Fig. 1). In contrast, the large hypoxic increase in SM-MHC abundance in adult arteries was also associated with decreased contractility, suggesting that simple abundance of SM-MHC is not a primary determinant of contractile capacity.

How hypoxic acclimatization selectively altered MHC isoform abundances remains uncertain but could be explained by either age-dependent hypoxic release of trophic factors or by increases in sensitivity to those factors. Whatever the nature of those factors, their effects on SM-MHC were uniform across the artery wall in both fetal and adult arteries, suggesting that these factors did not originate exclusively from either the

![Fig. 5. Effects of VEGF on MHC abundance and distribution. Endothelium-denuded carotid arteries from 6 normoxic fetal lambs and 6 normoxic adult sheep were serum starved for 24 h and then were cultured an additional 24 h in the presence or absence of 3 ng/ml VEGF. Following organ culture, the arteries were processed for immunostaining as described for Fig. 2. Organ culture with VEGF did not significantly (*P < 0.05) alter abundance for either MHC isoform except a modest increase of NM-MHC with VEGF in the inner medial region of fetal arteries. In both control and VEGF-treated fetal arteries, NM-MHC exhibited a gradient in which abundance was greatest in the inner medial layer and least in the outer medial layer. Shown are means ± SE for n = 6 for all groups.](http://ajpcell.physiology.org/doi/10.1152/ajpcell.00408.2011)
luminal or adventitial border of the medial layer. Overall, these results are consistent with previous reports that hypoxia can increase total MHC content (70) and also support the hypothesis that the artery wall contains smooth muscle in multiple phenotypic states with varying responses to trophic and mitogenic stimuli (24, 56, 69). These results, however, do not help explain hypoxic attenuation of contractility: how is it possible that hypoxia increased the content of SM-MHC but decreased contractility? Clearly, factors other than MHC abundance must be involved.

Smooth muscle contraction requires the interaction between myosin and SM-αA. SM-αA is a smooth muscle marker protein expressed in both immature noncontractile and fully differentiated contractile smooth muscle (25, 44). SM-αA abundance increases during maturation of contractile capacity (45) and also increases during hypoxic acclimatization (54). Because hypoxia can increase both SM-αA and MHC but simultaneously decrease contractile capacity, some aspect of the interaction between SM-αA and MHC must be affected by hypoxia. Whereas possible changes in numerous other smooth muscle proteins (59) might influence calcium handling, calcium sensitization, myosin phosphorylation, or cytoskeletal organization, virtually all of these mechanisms act by altering the activation of regulatory myosin light chain or its access to filamentous actin (57). Another possibility is that the spatial organization of MHC relative to SM-αA is altered as a consequence of structural responses to hypoxic acclimatization. To test this hypothesis, our experimental approach included measurements of the fraction of SM-αA colocalized with both of the main MHC isoforms using confocal microscopy as previously described (14). Colocalization of NM-MHC with SM-αA was significantly decreased by hypoxia in fetal arteries and was unchanged in adult arteries (Fig. 3) due perhaps to the very low content of NM-MHC in mature arteries. Conversely, colocalization of SM-MHC with SM-αA was decreased by hypoxia in adult but not fetal arteries. These results are consistent with the hypothesis that hypoxia increases the fraction of synthetic smooth muscle cells in the artery wall. In the fetus, hypoxia increased NM-MHC, decreased SM-MHC, and decreased colocalization of NM-MHC with SM-αA. These changes, together with no significant change in medial thickness (Table 1) and decreased contractility (Fig. 1), strongly suggest that in fetal arteries hypoxic acclimatization promoted a shift in smooth muscle characteristics toward a less contractile, more proliferative, more synthetic phenotype. In adult arteries, hypoxic acclimatization increased NM-MHC but also increased SM-MHC while decreasing colocalization of SM-MHC with SM-αA. The simultaneous increase in SM-MHC abundance with a decrease in SM-MHC colocalization with SM-αA suggests increased rates of contractile protein synthesis together with possible disassembly of the contractile apparatus. Again, these changes, together with no significant change in medial thickness (Table 1) and decreased contractility (Fig. 1), strongly suggest that in adult arteries hypoxic acclimatization...
also promoted a shift in smooth muscle characteristics toward a less contractile, more proliferative, more synthetic phenotype.

**VEGF altered artery structure and function.** Aside from the effects of hypoxic acclimatization on the abundance and organization of vascular contractile proteins and the corresponding changes in contractile capacity, it remains uncertain how chronic hypoxia might bring about such changes. One possibility is that VEGF may be involved. Secondary to hypoxic increases in hypoxia-inducible factor-I release (53), hypoxia stimulates the synthesis and release of VEGF, which promotes angiogenesis (28, 40) but can also exert trophic influences on many nonendothelial cell types including retinal pericytes (61), neurons (32), astrocytes (22), peripheral neurons and Schwann cells (39, 58), skeletal muscle (9), and most importantly, smooth muscle (11, 26, 43). To test the hypothesis that VEGF may contribute to hypoxic vascular remodeling, our experimental approach compared the effects of hypoxic acclimatization to the effects of organ culture with 3 ng/ml VEGF. This approach avoided the smooth muscle dedifferentiation typical in cultured smooth muscle (63) and preserved the three-dimensional structure of the artery wall required for colocalization studies. Our 3 ng/ml concentration of VEGF was similar to the physiological concentrations of 2 ng/ml reported in pregnant sheep (64) but was far less than the 40–50 ng/ml concentrations used in other studies of VEGF (5, 13), which helped minimize possible cross-activation of PDGF receptors that can occur at VEGF concentrations of 10 ng/ml or greater (4). To avoid the dedifferentiating effects caused in smooth muscle by exposure to FBS (34, 51), the organ cultures did not include FBS. Although organ culture can alter the abundances of some contractile proteins (11), such effects should similarly influence both stretch-dependent and K⁺-dependent contractions; these were measured in the same arteries. Because organ culture affected stretch-dependent and K⁺-dependent contractions very differently (Fig. 1, normoxic vs. Fig. 4, control), the results suggest that organ culture alters contractility through mechanisms other than just changes in contractile protein abundance. Given that peak contractile stresses independent of method of contraction were similar in fresh and organ cultured arteries, overall contractility was optimally preserved as previously described (11).

Similar to the effects of hypoxic acclimatization on contractility, organ culture with VEGF significantly decreased overall contractility in both fetal and adult arteries (Fig. 4). In adult arteries, this effect of VEGF was prevented by vatalanib, a mixed VEGF-R1/R2 antagonist (35) at a concentration (240 nM) validated to be optimal in this preparation. Vatalanib had no effect on VEGF-induced depression of contractility in fetal arteries. Together, these findings suggest that VEGF acts through VEGF-R1/R2 receptors to attenuate both stretch-dependent and K⁺-induced contractions in adult arteries. In fetal arteries, VEGF must act through a different pathway to influence contractility. The unique effects of VEGF on fetal arteries might possibly involve altered coupling of VEGF receptors to cytosolic kinases (42) but might also potentially involve activation of PDGF receptors (4) that are highly abundant in immature arteries (2); more experiments will be required to differentiate among these possibilities. Together, these results are consistent with the hypothesis that VEGF may contribute to hypoxic vascular remodeling through transformation of smooth muscle phenotype but also emphasize that the mechanisms involved must be very different in fetal and adult arteries.

**Effects of VEGF on MHC abundance and organization.** The general effects of VEGF on NM-MHC abundance were roughly similar to the effects of chronic hypoxia (Fig. 5, top right), although the effects of VEGF were more modest due perhaps to the low concentration of VEGF employed. As in normoxic fetal arteries, NM-MHC exhibited a graded decrease in abundance from the inner medial region to the outer medial region, suggesting a possible regional influence from the vascular endothelium. For SM-MHC abundance, however, VEGF did not reproduce the effects of hypoxic acclimatization and was without effect on either the abundance or the homogeneous distribution of SM-MHC throughout the media in both fetal and adult arteries. Despite its relative absence of effects on NM-MHC and SM-MHC, VEGF still decreased contractility in fetal and adult arteries, again suggesting a shift toward a less contractile smooth muscle phenotype.

In both fetal and adult arteries, hypoxia-induced changes in contractility were more closely associated with changes in contractile protein organization than with changes in abundance (Figs. 1–3). To examine if VEGF-induced changes in contractility were similarly associated with changes in contractile protein organization, we examined the influence of VEGF on the spatial organization of MHC isoforms relative to SM-α-A (Fig. 6). For NM-MHC, VEGF treatment decreased colocalization with SM-α-A in fetal but not adult arteries and thus replicated the qualitative effect of hypoxia. In addition, VEGF decreased colocalization of SM-MHC with SM-α-A in adult but not fetal arteries, which also reproduced the pattern produced by hypoxia. These colocalization results further support the hypothesis that VEGF may contribute to hypoxic vascular remodeling, at least in the ovine carotid artery. These results also reinforce the interpretation that contractility was more consistently associated with contractile protein organization.
tion than with contractile protein abundance; both hypoxia and VEGF similarly decreased contractility as well as NM-MHC colocalization in the fetus and SM-MHC colocalization in the adult. In contrast, hypoxia and VEGF produced only increases in the abundance of NM-MHC and had inconsistent effects on SM-MHC.

Another approach to compare the effects of hypoxic acclimatization and treatment with VEGF arises from the presumed obligatory relation between contractile protein abundance and organization. A protein can be colocalized with another protein only if both proteins have been synthesized and are present near one another. In other words, colocalization is limited by regional abundance. If colocalization is merely a stochastic process, then increased abundance of any protein should increase its colocalization with its binding partners. To test this idea, our data analysis compared parallel changes in abundance and colocalization following treatments with both hypoxia and VEGF. Altogether, these comparisons strongly suggested that the observed changes in colocalization were not simply secondary to increased abundance; in almost all instances the changes in abundance and colocalization were not directly proportional. This latter finding implies that both hypoxia and VEGF have significant and parallel effects on the abundances, trafficking, and localization of contractile proteins within arterial smooth muscle cells. The mechanisms mediating these effects are uncertain but could involve changes in cytoskeletal remodeling, microtubular transport, or protein-protein interactions as suggested in other cell types (3). Thus the present results follow a major trend in contemporary cell biology that is focused on the complex and unidentified mechanisms that govern subcellular protein distribution, trafficking, and localization.

Independent of mechanisms governing abundance and colocalization of smooth muscle contractile proteins, both hypoxia and VEGF appeared to recruit some of these mechanisms similarly. VEGF reproduced some, but not all, of the effects of hypoxic acclimatization, suggesting that factors other than VEGF must be involved in hypoxic vascular remodeling, particularly in relation to regulation of SM-MHC abundance. Conversely, hypoxia and VEGF had much more similar effects on colocalization of the MHC isoforms with SM-α-A in both fetal and adult arteries (Figs. 3 and 6). Interestingly, this close parallel between hypoxia and VEGF in relation to MHC colocalization was observed, even though VEGF may not signal through traditional VEGF receptor-dependent pathways in fetal arteries (Fig. 4). This concurrence of effects of hypoxic acclimatization and VEGF on MHC colocalization strongly supports the main hypothesis that VEGF may contribute to hypoxic vascular remodeling in both fetal and adult arteries.

Overview. Together, the results of the present study demonstrate that hypoxic acclimatization and VEGF exert both common and independent effects on contractility, MHC isoform abundance, and MHC-SM-α-A organization. In light of abundant evidence that hypoxia increases both VEGF (36, 37) and VEGF receptor density (8), the present findings suggest that hypoxic acts through VEGF not only to increase capillary density (28) but also to mediate remodeling of arterial smooth muscle (Fig. 7). The present results further emphasize that VEGF can act directly on vascular smooth muscle through receptor-dependent pathways that appear to involve VEGF-R1/R2 in adult but not fetal arteries; this difference may help explain many of the observed age-related differences in responses to both hypoxia and VEGF. Furthermore, these results also give rise to the hypothesis that smooth muscle phenotype is a determinant of the responses to hypoxia and VEGF and that one component of these responses may be to stimulate phenotypic conversion and thereby alter contractile protein abundance, organization, and function. On the other hand, differences in responses to hypoxia and VEGF emphasize that VEGF is not the only or perhaps even the most important vasotrophic factor contributing to hypoxic vascular remodeling. Many important questions remain: what other factors contribute; what receptors mediate fetal responses to VEGF; might larger concentrations of VEGF applied for longer durations produce greater or different effects; what mechanisms govern MHC colocalization independent of abundance? Aside from these and other questions for future investigations, the present results support the hypothesis that VEGF may contribute to hypoxic vascular remodeling in an age-dependent manner through effects that involve changes in MHC abundance, organization, and function.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

HYPoxia AND VEGF ALTER VASCULAR MYOSIN Isoform Profiles


