Structural inhomogeneity and fiber orientation in the inner arterial media

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Timmins LH, Wu Q, Yeh AT, Moore JE, Jr., Greenwald SE. Structural inhomogeneity and fiber orientation in the inner arterial media. Am J Physiol Heart Circ Physiol 298: H1537–H1545, 2010. First published February 19, 2010; doi:10.1152/ajpheart.00891.2009.—The microstructural orientation of vascular wall constituents is of interest to scientists and clinicians because alterations in their native states are associated with various cardiovascular diseases. In the arterial media, the orientation of these constituents is often described as circumferential. However, it has been noted that, just below the endothelial surface, the vascular wall constituents are oriented axially. To further study this reported change in orientation, and to resolve previous observations (which were made under conditions of no load), we used nonlinear optical microscopy to examine the orientation of collagen and elastin fibers in the inner medial region of bovine common carotid arteries. Images were obtained from this part of the arterial wall under varying degrees of mechanical strain: 0%, 10% axial, 10% circumferential, and 10% biaxial. We observed that close to the endothelium these components are aligned in the axial direction but abruptly change to a circumferential alignment at a depth of ~20 μm from the endothelial surface. The application of mechanical strain resulted in a significantly greater degree of fiber alignment, both collagen and elastin, in the strain direction, regardless of their initial unloaded orientation. Furthermore, variations in strain conditions resulted in an increase or a decrease in the overall degree of fiber alignment in the subendothelial layer depending on the direction of the applied strain. This high-resolution investigation adds more detail to existing descriptions of complex structure-function relationships in vascular tissue, which is essential for a better understanding of the pathophysiological processes resulting from injury, disease progression, and interventional therapies.

Regardles of such wide variation in findings, notable, yet often-overlooked, studies have acknowledged a distinct radial variation in fiber and cellular orientation within the medial layer. As first described by Wolkoff (47), the internal elastic lamina (IEL) of human coronary arteries appears to be split into two distinct laminae during growth and development. This process starts in utero by bending and fraying of the IEL, continuing through puberty and into old age. Gross et al. (13) reported similar findings in human coronary arteries in relation to aging. In particular, it was noted that as early as the third month postpartum, a splitting in the IEL occurs, leading to the formation of the “musculoelastic layer.” During the break in continuity of the IEL, VSMCs migrate into the newly formed layer and orient themselves axially, whereas those in the medial layer are still aligned circumferentially. It should be noted that during this process multiple elastin layers are formed; however, as reported by Gross et al., the outermost of the two elastin layers resulting from the split still represents the border between the intimal and medial layers and thus is still referred to as the IEL. In the present article, the term “subendothelial” refers to the region just below the endothelial surface but on the adluminal side of the IEL. Similarly, in the intracranial arteries of the rat and human, the IEL consists of a double layer, in this case associated with a honeycomb-like network of fine elastic fibers (48). IEL splitting and axially aligned fibrils have also been noted in pathological states, such as the formation of intimal pads or cushions and hypertension (39, 44). An axial orientation of inner medial fibers and VSMCs in the porcine aorta has been described by Clark and Glagov (6), who suggested that it is associated with shear stress from the flowing blood transmitted into the inner layers of the media. A similar arrangement has been seen in human cerebral arteries (37). Smith (38) described a variation in fiber orientation throughout the media of the rabbit pulmonary trunk; however, he noted that this variation was not seen in the aorta. Again, however, all observations were made under conditions of no mechanical load.

Blood vessels contain highly extensible, isotropic elastin fibers and strain-stiffening, anisotropic collagen bundles and have mechanical properties that are strongly dependent on the alignment of the embedded fibrous constituents. Recently, constitutive formulations that relate the applied stresses/forces to the resulting strains/deformations of biological tissues have shifted from purely phenomenological descriptions to those that are structurally based. These constitutive relations incorporate fiber alignment into their formulations to offer a better description of arterial mechanical properties (14, 52). Therefore, microstructural information, such as the density, interaction, and orientation of these vascular wall constituents, is essential if one wishes to accurately capture and model the material response of vascular tissue, both normal and diseased.

THE MICROSTRUCTURE of the normal arterial wall, while varying with species, location, age, and disease, comprises three layers: the tunica intima, the tunica media, and the tunica adventitia. In particular, the medial layer of conduit arteries consists of alternating layers of vascular smooth muscle cells (VSMCs) and elastic lamina (termed the musculoelastic fascicle), with collagen fibers interlaced between the elastic lamina (see Ref. 6, Fig. 11A). Numerous studies have examined the orientation of fibers and VSMCs within the media of conduit arteries, yet there has been no conclusive agreement on their preferred orientation. VSMC orientation has been expressed as circumferential (1, 8, 13, 46), oblique (2, 46), and helical (29, 46), with similar findings described for collagen and elastin fibers. However, we note that all observations were made on relaxed vascular tissue.

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Various optical techniques have been used to examine fiber orientation [e.g., histological polarized light microscopy (9, 45) and scanning electron microscopy (5, 22, 23)]. However, such methods are destructive to the tissue and are not suitable for examination of a single tissue sample under multiple experimental conditions. To overcome this limitation, nondestructive methods for the analysis of fibrous structures have been developed [e.g., small-angle light scattering (17, 21, 32) and small angle X-ray scattering (21, 25, 34, 35)]. Nonlinear optical microscopy (NLOM) provides an excellent means of examining the orientation and distribution of constituents within biological tissues. For instance, second harmonic generation [SHG (4)] and two-photon excited fluorescence [TPF (50)], both of which are confocal techniques, provide a means of collecting thin optical sections (i.e., images) from within thick specimens. Such attributes are ideal for a quantitative examination of fiber orientation under various experimental conditions and have been used in previous investigations (16, 30).

Our objective in this study was to quantify collagen and elastin fiber orientation in the inner part of the bovine common carotid artery using NLOM. In addition, analysis of the fiber alignment in this subendothelial layer was performed under varying degrees of mechanical strain, within the physiological range, as a means of resolving previous observations that were carried out under no-load conditions. The results from this investigation will provide further data on the relationship and interactions between arterial tissue components and thus lead to a better understanding of the structure-function relationships of vascular tissue in general.

MATERIALS AND METHODS

Following several reports of axially aligned fibers and cells on the abluminal side of the endothelial surface, we used NLOM methods to investigate this subendothelial region of the arterial medial layer. Optical sections were acquired radially outward from the endothelial surface with a custom-designed NLOM system. The orientations of extracellular matrix fibers were analyzed by an application of a subroutine based on a fast Fourier transform (FFT) algorithm and then quantified by a polar coordinate analysis of the filtered power spectrum of each image. NLOM results were qualitatively verified by comparing them to light microscopic images of histologically stained sections.

Tissue preparation and biaxial testing. Bovine common carotid arteries were collected from a local abattoir and transferred to the laboratory in PBS (18°C). After removal of loosely adherent perivascular tissue, an ~2.5-cm-long segment was removed, opened circumferentially with an axial cut, and attached to a modified version of a biaxial mechanical testing device as previously described (16). The remainder of the artery was set aside for histological evaluation. Briefly, the testing device consisted of four polycarbonate clamps sliding on stainless steel rods. The clamps and rods were arranged in pairs, and the pairs were oriented at right angles to each other. By moving either or both clamp pairs, independent movement in the x- and y-directions was possible. A square section (~2.5 cm × 2.5 cm × 2.0 mm) was cut from the artery for testing, and each edge was attached to a clamp by equidistant, noncontinuous loops of 2-0 silk suture. The device chamber was then filled with PBS (25°C) to completely submerge the tissue before it was imaged on the NLOM system (Fig. 1). Note that specimens were positioned such that the intimal surface was nearest the microscope objective (i.e., the first image acquired was of the endothelial surface). Specimen unloaded dimensions (0% strain state) were determined by stretching the tissue sample until it was flat and free of wrinkles, as determined by eye. Measurements of the distances between each pair of clamps were recorded and noted as the unloaded dimensions. Subsequent strain states (10% axial, 10% circumferential, and 10% biaxial) were achieved by sliding the stainless steel arms, thus moving the clamps to the measured locations and fixing their positions with set screws. Each pair of arms was moved a similar amount, ensuring that the center of the specimen did not move appreciably as it was stretched. All strain measurements were made with vernier calipers to the nearest 100 μm. The testing device, with the specimen intact, was then returned to the microscope stage, and images were acquired at the central location of the tissue specimen, as judged by eye.

NLOM. Our custom-built NLOM system has been previously described in detail (16, 20). Sub-10-fs pulses (800 nm, 133-nm full width at half-maximum) from a Kerr lens mode-locked Ti:Al2O3 oscillator (Femtosource, Femtolasers, Cambridge, MA) pumped by a frequency-doubled Nd:YVO4 solid-state laser (Verdi, Coherent, Santa Clara, CA) were coupled into the epifluorescence port of an upright microscope (Axioskop2 MAT, Carl Zeiss, Thornwood, NY) via dual-axis galvanometer-driven mirrors (Cambridge Technology, Lexington, MA) mounted on an elevated breadboard. The entire NLOM system was atop a vibration-isolated optical table. The laser beam was directed to the microscope focusing objective (×40 Achromplan, numerical aperture: 0.8, water immersion, Carl Zeiss) by a short-pass dichroic mirror (635dcxrvxv3p, Chroma, Rockingham, VT). Nonlinear optical signals were directed by the objective through one of two accessory ports on the binocular head to a custom-built dual-channel detector that housed two dichroic mirrors and band-pass filters (Chroma), focusing lenses (31 2321, Linos Photonics, Milford, MA), and a pair of photon-counting photomultipliers tubes (R7400P, Hamamatsu, Bridgewater, NJ). SHG and TPF signals were collected by appropriate long-pass dichroic mirrors (430dcxrvxv3p and 505dcxrvxv3p, respectively, Chroma) and band-pass filters (HQ405/40 and HQ480/40, respectively, Chroma). Photomultiplier tubes were connected to a preamplifier/discriminator (F-100T, Advanced Research Instruments, Golden, CO), which thresholds signal current and converts it to transistor-transistor logic pulses for photon counting. Due to the marked differences in spectral characteristics generated by collagen and other fluorescent tissue components (e.g., elastin), the SHG and TPF signal components were imaged and segmented simultaneously. The result was that SHG and TPF NLOM images (optical sections) of fibrillar collagen (types I and III) and elastin in the media, respec-
tively, were acquired in the central region of the specimen. The optical signal-to-noise ratio was improved by capturing four images at a rate of 0.5 Hz/image and averaging them. Successive averaged images were obtained starting from the intimal surface and moving medially in 0.5-μm steps to 200 μm deep into the tissue. They were saved as raw 16-bit data files. Throughout the set of experiments described in the present study, <40 mW of laser power was incident on the scanning mirrors, thus minimizing thermal effects.

**Histology.** Standard histological techniques were used to process the arterial segments. Briefly, specimens were fixed in formal saline for 24 h and embedded in paraffin wax, and transverse and longitudinal sections were cut at 3 μm. Sections were then mounted on electrostatically charged slides to enhance the adhesion of the tissue to the glass (Superfrost Plus, VWR), dried, and stained either with Ehrlich's hematoxylin (11), an adaptation of Miller's elastin stain (24), or picrosirius red (41) for the visualization and examination of cell nuclei, elastin, and collagen, respectively.

**Image processing, analysis, and evaluation.** Before image acquisition, the focal plane was set just above the surface of the specimen (~5 μm). This setting ensured a clear signal from the luminal surface of the intima as the image plane was moved deeper into the arterial wall (i.e., outward in the radial direction). After image stack acquisition (SHG and TPF), a Matlab (MathWorks, Natick, Massachusetts) subroutine was developed to determine the location of the intimal surface. Briefly, the average intensity value was determined for each SHG image in the stack, and the image with the maximum average pixel intensity was determined. The innermost image (i.e., closest to the endothelial surface) in the stack that had a mean intensity value ~30% of the maximum average pixel intensity was defined as the location of the intimal surface, and data analysis of both SHG and TPF images was carried out from that point. Sensitivity analysis indicated that mean intensity values of ~20% and 40% of the maximum average pixel intensity resulted in a 3.5- and 2.5-μm shift, respectively, in the location of the image within the specimen thickness. Compared with the differences in cross-over region location (<1 μm), this was deemed an acceptable justification of the choice of 30% intensity as the inner wall threshold.

After the location of the intima, images (400 images/stack, maximum depth in the media: 200 μm) were analyzed in a modified version of the previously described Matlab (MathWorks) subroutine to analyze collagen and elastin fiber orientation (16). Raw image data files were padded with redundant data to enhance frequency resolution. Padding was achieved by reflecting (mirroring) the original image at 0, 90, 180, and 270° (Fig. 2B) and applying a Gaussian decay (SD: 0.25) to the reflected images, such that the pixels bordering the image had a <0.04% reduction in the original pixel intensity and those at the edges were reduced to zero (i.e., pixel value of 0, black). The padding increased the size of the image from 237 × 256 to 512 × 512 pixels. Note that due to acceleration of the horizontal scanning mirror during the start of each scan line, the first 19 columns of pixels were removed. To reduce edge effects, a two-dimensional Hann window function was applied, followed by transformation of the image to the frequency domain (i.e., power spectrum) using a two-dimensional FFT (Fig. 2C) (5). High- and low-pass Butterworth filters (sixth order) were applied, removing frequencies >60 Hz and <6 Hz. These cutoff frequencies were chosen by examination of the resulting inverse FFT images after filtering. Evaluation of the angular distribution of fibers was done by polar coordinate analysis of the filtered power spectrum image as described by Nishimura et al. (27). Briefly, the relative intensity (RT) for angles (θ) ± 2° was calculated as follows:

\[
RT(\theta) = \frac{1}{N} \sum_{i=1}^{N} |F(\theta_i)|^2
\]

where N is the number of pixels in the ROI (region of interest), and F(θ) is the magnitude of the filtered power spectrum at angle θ. The alignment index (AI) was calculated as the horizontal shift of the peak fiber angle (PFA) in the frequency histogram (D) to determine the predominant fiber angle (PFA) and calculate the alignment index (AI) (30). This algorithm was applied to all images within the optical stacks.

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*Fig. 2. Algorithm to calculate the fiber angle from NLOM images. The original NLOM optical section (A) was padded, and a windowing function was applied (B). A two-dimensional fast Fourier transform was then performed to obtain the power spectrum (C), which allows for the generation of the frequency histogram (D) to determine the predominant fiber angle (PFA) and calculate the alignment index (AI) (30). This algorithm was applied to all images within the optical stacks.*

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1 Average pixel intensity and maximum average pixel intensity were also determined for the TPF images in each image stack; however, the differences between the SHG and TPF maximum average pixel intensity locations were small (2.5 ± 1.0 μm) with no noticeable trend. Thus, only the SHG images were used to determine the location of the intimal surface.
bovine carotid artery specimens under no strain revealed a fluorescence (TPF; elastin, red) images within a representative arterial specimen from the cross-over region and deemed significant. All results are reported as means ± SE.

RESULTS

Examination of the NLOM images in the central region of bovine carotid artery specimens under no strain revealed a sudden change in fiber orientation ~20 μm below the endothelial surface. Both collagen (SHG signal) and elastin (TPF signal) fibers shifted from a predominantly axial orientation (90°) to a largely circumferential (0°) orientation (Fig. 3).

Examination of the PFA values for all strain conditions revealed a sharp change in collagen fiber angle from an axial orientation just below the endothelial surface to a circumferential orientation deeper in the medial layer. Furthermore, this shift in fiber angle can be seen by eye in the NLOM images. It was observed that in the optical sections 5 μm inward from the cross-over region (i.e., 5 μm toward the intimal surface), the fibers were aligned predominantly in the axial direction (vertical), whereas the optical section 5 μm outward from the cross-over region (i.e., 5 μm toward the adventitial surface) revealed fibers aligned predominantly in the circumferential direction (horizontal).

Statistical analysis. Mean PFAs and AIs were calculated for each specimen (n = 4) under four mechanical strain conditions. Differences between the orientation parameters under each mechanical state were determined using a paired Student’s t-test, with P values of <0.05 deemed significant. All results are reported as means ± SE.

2 Results from SHG (collagen fibers) and TPF (elastin fibers) data were not significantly different; thus, for brevity, only the SHG data are shown in Figs. 3 and 7.

![Fig. 3. NLOM images under the various strain conditions. Shown are combined second harmonic generation (SHG; collagen, green) and two-photon excited fluorescence (TPF; elastin, red) images within a representative arterial specimen from the cross-over region and ±5 μm from that position at each of the four strain conditions. (Note that the orange-yellow color shows regions giving rise to SHG and TPF signals.) The radial positions of the cross-over images are shown by the open circles on the alignment index plots (bottom right; SHG data only), and the solid circles correspond to the positions ±5 μm from the cross-over location. It was observed that fibers ~5 μm inward from the cross-over region (i.e., toward the endothelial surface) were aligned predominantly in the axial direction (vertical, 90°), whereas fibers +5 μm outward from the cross-over region (i.e., toward the adventitia) were oriented circumferentially (horizontal, 0°). Such observations were further supported by an evaluation of PFA as a function of radial position (top right, SHG data only). Scale bar = 50 μm. It should also be noted that fenestrations in the elastin lamellae were observed, further supporting the use of NLOM to observe the microstructure of vascular tissue.](http://ajpheart.physiology.org/)
observed when we examined the elastin fiber orientation, as PFA values of $82 \pm 4.3$, $87 \pm 1.9$, $75 \pm 9.6$, and $80 \pm 6.2^\circ$ were calculated for the optical sections section $5 \, \mu m$ inward for the four strain conditions, respectively, and optical sections $5 \,\mu m$ outward had statistically significant changes ($P < 0.01$) in PFA values of $8.0 \pm 2.0$, $13 \pm 4.7$, $3.0 \pm 1.0$, and $6.0 \pm 1.6^\circ$ for the four strain states, respectively (Fig. 4 and Table 1). It should be noted that the radial positions of the cross-over regions differed in the SHG and TPF optical sections of each specimen; however, the differences were small ($5.8 \pm 1.5 \,\mu m$), with no noticeable pattern in the four specimens studied.

Histological evaluation provided a larger field of view of the arterial specimens and further qualitative evidence that confirmed the observations determined from the NLOM images. Transverse and longitudinal sections revealed a region just below the endothelial surface where there was a sharp change in VSMC nuclear orientation that coincided with a change in fiber (collagen and elastin) structural organization in the artery wall (Fig. 5). Examination of the micrographs stained with Ehrlich’s hematoxylin revealed VSMC nuclei aligned predominantly axially in the subendothelial region, followed by an abrupt shift as the VSMCs assumed a circumferential orientation deeper in the arterial medial layer (Fig. 5, A and D). Note that in transverse histological sections, cell nuclei and fibers appeared nearly circular if aligned axially, whereas they were elliptical and elongated if aligned in the circumferential direction. Conversely, in longitudinal sections, they were elliptical and elongated if oriented axially and circular if aligned circumferentially. When we examined the elastin fibers, a discontinuity in their appearance was seen at a similar radial position (Fig. 5, B and E), although in these sections one cannot say with confidence that the orientation of the elastic tissue changed. This discontinuity was more clearly evident in the longitudinal section (Fig. 5E), as moving outward radially revealed a sudden change in the elastin structure just below the endothelial surface. A similar finding was observed when we examined the collagen fiber structure in the sections stained with picrosirius red (Fig. 5, C and F).

The radial position of the abrupt change in fiber orientation depended strongly on the direction of the applied strain when we compared the four mechanical states (0%, 10% axial, 10% circumferential, and 10% biaxial). Applying a 10% axial strain led to a significant inward shift (i.e., movement closer to the endothelial surface) in the location of the cross-over region compared with the unloaded case ($P < 0.05$; Fig. 3). The fractional change in the distance from the intima to the cross-over region was $0.62 \pm 0.13$ for collagen (SHG images) and $0.60 \pm 0.10$ for elastin (TPF images) when normalized to the distance to the cross-over region in the 0% strain state (Fig. 6). A 10% biaxial strain also resulted in a significant inward shift in the radial location of the cross-over region when normalized against the 0% case (collagen: $0.50 \pm 0.08$ and elastin: $0.57 \pm 0.08$, $P < 0.01$). In contrast, application of a 10% strain in the circumferential direction revealed a tendency toward an outward shift in the radial location of the cross-over region;

### Table 1. Collagen and elastin fiber orientations near the cross-over region

<table>
<thead>
<tr>
<th></th>
<th>Collagen (SHG)</th>
<th>Elastin (TPF)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0%</td>
<td>10% Axial</td>
</tr>
<tr>
<td><strong>Subendothelial region</strong> (−5 μm from the cross-over region)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFA,°</td>
<td>83 ± 4.4</td>
<td>86 ± 2.3</td>
</tr>
<tr>
<td>AI</td>
<td>1.66 ± 0.03</td>
<td>2.03 ± 0.23</td>
</tr>
<tr>
<td>PFA,°</td>
<td>82 ± 4.3</td>
<td>87 ± 1.9</td>
</tr>
<tr>
<td>AI</td>
<td>1.70 ± 0.06</td>
<td>1.91 ± 0.12</td>
</tr>
<tr>
<td><strong>Inner medial region</strong> (+5 μm from the cross-over region)</td>
<td></td>
<td></td>
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<tr>
<td>PFA,°</td>
<td>7.0 ± 3.8</td>
<td>10 ± 2.8</td>
</tr>
<tr>
<td>AI</td>
<td>1.98 ± 0.05</td>
<td>1.77 ± 0.15</td>
</tr>
<tr>
<td>PFA,°</td>
<td>8.0 ± 2.0</td>
<td>13 ± 4.7</td>
</tr>
<tr>
<td>AI</td>
<td>1.73 ± 0.13</td>
<td>1.77 ± 0.18</td>
</tr>
</tbody>
</table>

Data are means ± SE; $n = 4$ tissue samples. SHG, second harmonic generation; TPF, two-photon excited fluorescence; PFA, predominant fiber angle; AI, alignment index.
Fig. 5. Histological sections of the bovine common carotid artery. Shown are representative micrographs (A–C: transverse; D and E: longitudinal) from a test specimen stained with either Ehrlich’s hematoxylin (A and D) (11), an adaptation of Miller’s elastin stain (B and E) (24), or picrosirius red (C and F) (41) to visualize cell nuclei, elastin, or collagen orientation, respectively. Axially oriented VSMCs and extracellular matrix fibers were clearly observed just below the intimal surface, with an abrupt shift to a circumferential orientation deeper in the arterial wall. Note that the specimens were fixed in the unloaded state. The distance from the intima to the cross-over region was determined for each optical section (SHG and TPF) at each loading condition.

Fig. 6. Thickness of the axially oriented subendothelial region normalized to the unloaded state. The distance from the intima to the cross-over region was determined for each optical section (SHG and TPF) at each loading condition. Straining the specimens 10% axially or biaxially resulted in a reduction of the thickness of the subendothelial region relative to the unloaded condition. However, application of a 10% circumferential stretch to the specimens resulted in a slight, although not significant, thickening of the region. Data are means ± SE of 4 tissue samples. *Significant differences (P < 0.05); **significant differences (P < 0.01).

Fig. 7. Histograms of the percentage of pixels aligned in the longitudinal direction at 4 optical sections (SHG and TPF) for the unloaded condition (0 mmHg). Scale bar = 100 μm. L, lumen.

DISCUSSION

The purpose of this investigation was to examine the collagen and elastin fiber orientation in the subendothelial region of bovine common carotid arteries. High-resolution images were acquired using a custom-built NLOM system allowing for the analysis and quantification of extracellular matrix fiber orientations under varying levels of strain for, we believe, the first time. The results indicate that the fiber orientation and alignment are highly dependent on the direction of the applied strain and the direction in which the fibers are initially oriented.

The application of strain to vascular tissue leads to the reorganization of collagen and elastin fibers within the vascular wall, as the constituents bear the applied loads. Our results indicate that higher axial fiber alignment (increased AI values; Fig. 7 and Table 1) is a result of straining the tissue, however, only if the strain is applied in the axial direction. In contrast, if circumferential strain is applied, the fibers become less aligned (decreased AI values), an indication that the fibers may be realigning in the direction of the applied strain. Such results are consistent with findings from other investigations of the fiber orientation of vascular tissue under mechanical strain. Schmid et al. (35) used small-angle X-ray scattering to demonstrate that collagen fibers reorient themselves in the direction of applied stretch. Moreover, macroscopic force measurements were acquired and indicated that collagen fiber reorientation is only dependent on strains that exceed 4% (i.e., in the heel region of the stress-strain curve). Similar results have also been observed in other biological tissues, as Lake et al. (19) demonstrated that uniaxial loading of the supraspinatus tendon led to significant collagen realignment in the direction of the load throughout various regions of the tissue. Furthermore, the mechanical response of the tissue was highly dependent on both the unloaded and loaded fiber configurations (e.g., higher initial alignment corresponds to stiffer mechanical response). While the stress-strain relationship was not investigated in this
study, our data suggest that the nonlinear, anisotropic mechanical response for vascular tissue is a direct result of fiber orientation (10, 14). This strongly supports the suggestion of Clark and Glagov (6) that the relatively thin subendothelial layer is arranged so as to support loads that are in the axial direction (e.g., axial shear stress from blood flow), whereas the thicker, predominantly circumferentially aligned fibers deeper in the vascular wall bear the load of the pulse pressure waves during the cardiac cycle.

There is a strong correlation between the underlying microstructure of a biological tissue and its mechanical properties. For example, it has been shown that tissues are stiffer in the direction that the embedded matrix fibers are aligned [e.g., arteries (10, 14) and tendons (19)]. Applied loads lead to local deformations in the microstructure, resulting in macroscopic changes (thickening or thinning) of the tissue. For the axial and biaxial strain states, a thinning of the subendothelial axially aligned fiber layer was observed; however, a trend toward thickening was noted when a circumferential strain was applied (Fig. 6). Such an observation would indicate a negative Poisson’s ratio or auxetic behavior, which, although not generally accepted for vascular tissue, has been reported (42). More importantly, rather, this result further emphasizes the complex effects of fiber anisotropy on the mechanical properties of biological tissues. When fibers are oriented in the direction of the applied strain, they bear more of the load or are more stressed, resulting in a thinning of the material. However, if the material is multilayered with fibers not oriented in the direction of the applied strain, as investigated in this study, then such fibers will bear a minimal proportion of the applied load. Therefore, the stress in this (these) layer(s), being substantially smaller, does not result in a thinning of the layer.

The results further confirm the presence of a subendothelial layer of extracellular matrix fibers and VSMCs that are aligned longitudinally in vascular tissue. While we examined only the bovine carotid artery, we have seen structural inhomogeneity in the conduit arteries of other species, such as the pig (40). Explanations for the presence of this layer have been directed toward the complex, in vivo mechanical environment that is imposed on the vasculature. It has been suggested that endothelial cells on the intimal surface transmit the forces associated with the axial blood flow into the inner medial region (6). As the axial shear stress in the artery wall decays rapidly with radial position, only a small region of the media is subjected to this component of stress, whereas the remainder of the media is under circumferential stress due to the cyclic loading of pressure, resulting in circumferential alignment. In human common carotid arteries, typical values of flow-induced axial wall shear stress and pressure-induced circumferential wall stress are ~0.7 Pa and 75 kPa, respectively, at systole (7, 18). Thus, when examining the applied physiological loads, the minimal thickness of the subendothelial region compared with the thicker circumferentially aligned region of the media is understandable. Furthermore, it might be expected that if the blood flow rate is increased while holding the vessel caliber constant (i.e., increased mean shear stress), then the resulting shear forces would be transmitted more deeply into the medial layer, resulting in a thicker subendothelial axially aligned region. Measurements in situations in which mean shear stress is raised, such as downstream from a stenosis or arteriovenous shunt, would provide a test for this prediction.

If shear stress is indeed the principal reason for the axial alignment in the components of the inner media, the following question arises: Why is the transition at its outer edge so abrupt? If it is assumed that the axial tension due to shear stress falls gradually with increasing depth into the media, then a similar gradual change in orientation might be expected. On the other hand, if a threshold level of stress is necessary to provoke a change in orientation, this could give rise to the sudden change observed. We note that a radial gradient of circumferential stress may also be present in the inner media despite the existence of residual stresses (which also vary

Fig. 7. Collagen fiber alignment under the various mechanical strain conditions. Analysis of the collagen fiber distribution and alignment indexes (AI) (30) from the optical sections −5 μm (solid symbols) and +5 μm (open symbols) from the cross-over region revealed the effects of applying varying degrees of strain along orthogonal axes. Application of a 10% axial or biaxial strain resulted in higher AI values for the axially aligned subendothelial region (−5 μm) compared with the 0% strain state (closed symbols in the right graphs). However, a 10% strain in the circumferential direction decreased the AI value in that region. The AI values for the circumferentially oriented regions (+5 μm) decreased when a 10% strain was applied in the axial or circumferential direction, yet the value increased when a 10% biaxial strain was applied. Note that analysis of the TPF (elastin) images (not shown) showed similar findings; 90° = axial direction and 0°, 180° = circumferential direction. Data are means ± SE of 4 tissue samples.
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Across the arterial wall (12). A definitive test of these speculations would require measurements and/or modeling of the balance between pressure-induced circumferential stress and flow-induced shear stress (both applied and residual) but could provide valuable insight into the mechanisms of mechanotransduction in endothelial cells and VSMCs both under normal and pathological conditions.

As the NLOM techniques allowed for the simultaneous, independent examination of collagen and elastin fibers, the relationship between these two matrix proteins within the arterial wall could be further investigated. There were no statistical differences between collagen and elastin fiber orientation data for all strain states, thus supporting the notion of the interrelated architecture between these two extracellular matrix proteins, as has been described in the main body of the medial layer (6, 28).

We note a number of potential limitations to the study. First, although NLOM provides an excellent means for examining the microstructure of biological tissues, the submicrometer resolution of this technique results in image areas that are quite small (~0.02 mm²). Thus, it is likely that local heterogeneities within the tissue sample would not be detected. While not presented, analysis of optical sections at various locations on the tissue specimens revealed no significant changes in the depths of the cross-over region or the PFA and AI values. Second, some variability among specimens from different animals was also observed. However, as the error bars in Fig. 4 (for instance) show, these were acceptably small. Third, it was assumed that images were acquired at the exact middle of the specimen after its removal from the microscope stage and subsequent replacement when changing the strain state. However, as discussed above, the small variation of the measured orientation variables within each specimen ensured that any small deviation from this middle position did not confound the results. Fourth, in any optical study that analyzes structural orientation, polarization of the incident light may bias the results. In this case, a comparison of values between a tissue sample mounted in the device chamber and orientated normally on the microscope stage and one where the chamber was rotated 90°, both at the same strain state, was performed. Analysis of these results showed no statistical difference in any of the measured variables. Fifth, collagen types I, III, and V are present in the medial region of vascular tissue; however, SHG signals only result from fibrillar collagens (types I, II, and III) (33, 49). Collagen type V is only present in the basement membrane on the endothelial surface and VSMCs and does not affect the overall mechanical response of vascular tissue. In contrast, collagen types I and III, which have been shown to colocalize in human vascular tissue, are noted as the major load-bearing forms of this protein in the conduit arteries and thus affect the overall response to mechanical loads (3, 15, 36). Finally, due to the limited penetration depth of the NLOM signals, this study did not analyze the fiber orientation through the entire thickness of the artery wall. In particular, the high turbidity of vascular tissue results in significantly reduced image quality at depths >200 μm. However, in the 200 μm that were analyzed, high-resolution images of high quality were acquired, allowing for the visualization and quantification of the abrupt shift in fiber angle that had been previously noted.

Complete analysis of the microstructure of thick arterial specimens (>200 μm) will require a thorough description of the orientation and organization of cellular and matrix proteins. To this end, we have developed methods to mechanically remove circumferential layers of a known thickness (12, 43). This technique would allow serial examination of the entire thickness of any size artery.

In conclusion, this study provides further insight into structure-function relationships within vascular tissue. The results not only reveal the native fiber and cellular organization but also their reorganization and realignment under various mechanical strain states. The methods presented to assess fiber orientation could assist in bridging the gap between the microscopic and macroscales, allowing for a more informed incorporation of microscopic structural information into material models of biological tissues, as described by Sacks (31), while the presented NLOM imaging techniques could prove to be a powerful tool in advancing the understanding of vascular biomechanics, as noted by Zoumi et al. (51). The data presented here may also be of use to enhance the design of tissue-engineered cellular scaffolds for vascular grafts that directly mimic native vessel architecture.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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4 Now reformed as the Department for Business, Enterprise, and Regulatory Reform.

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