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Sustained Axial Loading Lengthens Arteries in Organ Culture

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Abstract—Although it has been recognized for many years that arteries *in vivo* exist under significant axial strain, studies of the adaptation of arteries to elevated axial strain have only recently been conducted. To determine the effects of sustained elevation of axial loading on arterial structure and function, axial stresses of 250 kPa or greater were applied to porcine common carotid arteries maintained in a perfusion organ culture system for 7 days at physiologic pressure and flow conditions. Our results demonstrated that axial stretch could lead to an increase in unloaded length that was proportional to the axial stretch ratio (stretched length divided by unloaded length) when the axial stretch ratio was above a threshold value of 2.14. Below this threshold, no significant length change occurred. Above this threshold, a significant increase in unloaded length ($13 \pm 7\%$,) and the number of smooth muscle cell nuclei ($20 \pm 7\%$) was observed. Permanent length change was associated with a significant decrease in axial stiffness, and the maximum elongation achieved was limited by rupture of the arterial wall. All tested arteries demonstrated good viability and strong vasomotor responses. These results show that arteries in organ culture can elongate under sustained axial loading.

Keywords—Blood vessels, Smooth muscle, Mechanical stimulation, Perfusion system, *ex vivo*, Adaptive response.

INTRODUCTION

Arteries *in vivo* are normally in a state of axial stretch (strain) that varies with age and location in the vascular tree.[14,27] The axial stretch consists of a large mean component with time-varying components resulting from pulsatile blood pressure and body movements such as beating of the heart or flexing of the joints.[19] It is widely acknowledged that arteries respond to mechanical stress and strain, but most of the related data available are limited to the adaptation of arteries to circumferential stretch produced by blood pressure.[26,29,33,38] Although recent studies suggest that variation in axial stress may lead to changes in arterial structure and function,[7,24] little is known about arterial adaptive or maladaptive responses to axial stretch.

Studies of axial stretch of arteries *in vivo* using implantable tissue expanders[8,12,36] or bone-lengthening external fixators[11,18,23] have been reported. Arteries remained patent during all but the most rapid applications of axial stretch,[36] and even the rapidly stretched arteries regained patency once the strain applicator was removed. Though gradually applied strain produced less arterial damage than rapidly applied strain, in general arteries tolerated these axial stimuli well. However, the applied strain and strain rate were not clearly defined in these reports due to the methods used to apply the loading.

More recently, rabbit carotid arteries were shown to return to normal strain levels 7 days after the application of elevated axial strain *in vivo*.[24] The adaptive response was characterized by a 15-fold increase in the rate of smooth muscle cell proliferation. These results clearly demonstrated the ability of arteries to rapidly lengthen, but the reported axial stretch protocol did not fully explore the possible maximum lengthening response.

There is a need for viable vascular grafts in coronary and peripheral vascular bypass applications. Arteries are by far the most desirable grafts due to their superior long-term patency, but there is a limited supply of arterial tissue.[3,10] Lengthening a suitable artery may provide a means to increase the supply of available tissue.[7,17] However, the effects of axial stretch on arteries need to be more fully understood.

Perfusion organ culture of intact artery segments provides an experimental system in which the effects of well-defined mechanical stimuli on vascular biology can be studied.[1,2,4,15,25,28,30] In this system, constituent cells remain in their native matrix architecture in a simulated hemodynamic environment and remain viable for over 7 days.

The objectives of this study were to determine the limits of axial strain for porcine carotid arteries in perfusion organ culture and to determine the effect of that stimulus on vascular structure and function.

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METHODS

Experimental System

The organ culture system, shown schematically in Fig. 1, was used to culture arteries for a period of 7 days. This system, modified from that described by Han and Ku[15], can apply computer-controlled axial stretch to arteries during culture. It consists of a polycarbonate artery culture chamber, tubing (Cole-Parmer, Phar-Med), and a roller pump (Cole-Parmer, Masterflex) that circulates cell culture medium. Pump speed and tubing resistance were adjusted to produce a sinusoidal pulsatile pressure of 100 ± 20 mmHg and a mean wall shear stress in the pressurized and stretched arteries of 15 dyn/cm^2. Physiologic temperature (37°C) was maintained by housing the entire system in a cell culture incubator (Isotemp, Fisher Scientific).

Arteries were mounted to two thin-walled stainless steel cannulas that approximately matched artery inner diameter (4.2 mm). The stationary cannula was fixed to the culture chamber by tight fittings while the sliding cannula could move axially through a port fitted with a custom-designed Teflon seal (American Variseal Corporation). A linear motor (LinMot), which included an integral displacement sensor, controlled the displacement of the sliding cannula and hence the stretch applied to the cultured artery. The position of the linear motor was measured and controlled using a PC. The axial force applied to the arteries was measured using a 4.5-kg force transducer (Sensotec).

Perfusion and bathing media were DMEM (Sigma, D1152) with 10% newborn calf serum (Hyclone, SH30072.03), and the medium pH was maintained by gas exchange with a 5% CO_2/95% air mixture. Dextran (Sigma, D4876) was added at a concentration of 6.5% to approximate the viscosity of blood (4 cP).

Arteries

Bilateral porcine common carotid arteries were harvested from 6- to 7-month-old farm pigs (115–135 kg) at a local slaughterhouse, rinsed with phosphate buffered saline (PBS, Sigma, D5652), and transported to the laboratory in ice-cold PBS. Perfusion circuit components were sterilized by autoclaving. Segments of the left and right carotid arteries, one serving as test specimen and one as control, were prepared for organ culture inside a laminar flow hood by trimming away the outermost layers of loose connective tissue, identifying leaks by brief inflation with air from a syringe, and tying off-side branches as necessary. Each artery segment was trimmed to approximately 5 cm and the no-load length of the segment was measured with an accuracy of ±0.05 mm using a video micrometer. Artery segments were mounted to the cannulas using one suture on each end with the *in vivo* flow direction preserved. Prewarmed media were added, and the mounted arteries were restored to their no-load lengths. The distance between the sutures was measured for each artery and used as the no-load gage length.

Stretching Protocols

Axial loading was applied to the arteries using specified protocols and software written in the C programming language for closed-loop control of stretch. During axial loading, arterial lengths (L) were determined using the motor position (20-μm resolution) and the initial no-load gage lengths (L_i). The axial stretch ratio, λ_z, for each artery segment was computed as

$$\lambda_z = \frac{L}{L_i} \tag{1}$$

The corresponding axial stress, σ_z, in the artery was computed on the basis of the Cauchy stress formulation:

$$\sigma_z = \frac{F\lambda_z}{A_i} \tag{2}$$

where F is current axial force and A_i is the initial no-load cross-sectional area of the artery measured before culture from calibrated video images of backlit artery rings approximately 2 mm long.

To explore arterial adaptation to axial loading near the rupturing threshold, two stretching protocols were used. Ruptures were repeatedly observed in arteries at axial

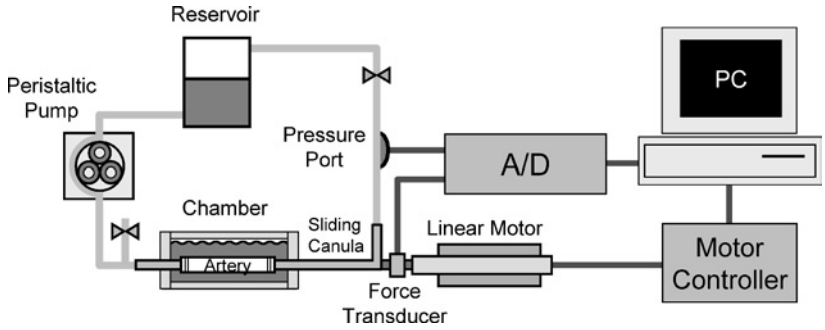

FIGURE 1. Schematic of the perfusion organ culture system. Axial stretch was applied to cultured arteries through the sliding cannula using the linear motor. Closed-loop control of the applied stretch was provided by a PC running custom C-code.

stresses of 1000 and 500 kPa, but not at 250 kPa. Therefore, an isotonic-type load of 250 kPa was selected for Protocol 1 to provide a sustained stimulus, and this load was applied throughout the 7-day culture period. During culture, the axial stretch ratio and stress were computed every 6 s, and the artery length was increased in 20-μm increments as necessary to maintain the desired axial stress. Protocol 1 represents a more intense stimulus than that used in earlier experiments where a constant stretch ratio of 1.8 was applied.[16]

When Protocol 1 did not yield significant length increase in these arteries, a more intense axial stimulus was sought to estimate the maximum axial loads the arteries could withstand without rupturing. However, simply increasing the load in Protocol 1 proved problematic, due to artery variability. At a given stress value, some arteries failed to show permanent length increase while others ruptured. On the other hand, the levels of axial strain at rupture were more consistent than the stress loads. Limiting the maximum axial stretch ratio to the range of 2.3–2.4 prevented rupture in most of the arteries.

Therefore it was decided to use an isometric-type stimulus in Protocol 2. Maximum stretch ratios in the range of 2.3–2.4 were used in Protocol 2, and this generally prevented rupture. Stresses between 350 and 650 kPa were applied to the arteries to gradually reach the target stretch range, usually within 2 days. Once a stretch ratio in this range was reached, artery length was maintained for the remainder of the 7-day culture period, and the axial load progressively decreased due to stress relaxation.

Control arteries were gradually extended to their *in vivo* length, an axial stretch ratio of 1.5, during the first 1–3 h and maintained at this stretch ratio throughout the culture period.[15] Separate control groups were used for Protocols 1 and 2 to minimize possible individual differences.

After culture, arteries were removed from the cannulas and placed in warm cell culture medium for 30–60 min to permit full viscoelastic relaxation. Definite grooves remained in the arteries where the sutures had been, and the distance between the grooves was measured in the no-load state using the video micrometer system. This distance was the postculture no-load gage length.

Axial Force-Length Response and Axial Moduli

To examine the mechanical properties of the arteries before and after organ culture, medium perfusion was stopped and arteries were returned to an axial stretch ratio of 1.5 relative to the initial no-load gage length. Arteries were preconditioned with 4–5 cycles of axial stretch between $\lambda_z = 1$ and 1.8 at 0 mmHg while they were still mounted in the culture chamber. The passive axial force-length response of each artery segment was measured over the same range of stretch ratios at an average strain rate of 0.001 s^{-1}. Tests were conducted at constant transmural pressures of 0 and 100 mmHg using a syringe pump to maintain pressure within the lumen. Artery length was measured using motor position, and the outer diameter was simultaneously measured from video images calibrated against a standard of known diameter.

Axial force-length data were acquired at the beginning and at the conclusion of the 7-day culture period for each artery in Protocol 2. Axial force-length data were not acquired in the initial group of arteries in Protocol 1 because the equipment was not in place. Additional experiments were subsequently conducted to measure the axial force-length response for Protocol 1. In all experiments, average frictional force on the sliding cannula and average axial pressure force within the artery were subtracted from axial force data before converting to axial Cauchy stress. Length data were converted to axial stretch ratios based on the unloaded length measured at the time of testing (i.e., distinct unloaded lengths were considered for the same artery before and after culture). The stress–strain data were fit using exponential curves. Incremental axial Young's moduli were calculated from the curve slopes at axial stretch ratios of 1.5, which is the axial stretch ratio *in vivo*, and 1.65.

Vasomotor Response

Vasomotor response of the artery segments after 7 days in culture was measured as the change in outer diameter of the arteries due to the action of norepinephrine (NE, Sigma) and sodium nitroprusside (SNP, Sigma). Arteries were perfused at 100 ± 20 mmHg and tested at an axial stretch ratio of 1.5 based on the initial no-load length. Diameter measurements were made at a sampling frequency of 1 Hz. NE and SNP were added sequentially to the perfusion medium at 30-min intervals at final concentrations of 1×10^{-6} M. The response for each drug was calculated as the change in diameter divided by the initial baseline diameter measured immediately before vasomotor testing. The diameter was defined as the time-average of all diameter measurements from the 2-min interval preceding the time point of interest.

Histology

Tissue samples were fixed overnight in 10% neutral buffered formalin. Samples were then processed for paraffin embedding. Five-micrometer sections were cut and stained using either hematoxylin and eosin or the staining protocols described below.

Cell proliferation was detected using 5-bromo-2′-deoxyuridine (BrdU, Sigma) incorporation. BrdU, at a final concentration of 5 mg/l, was added to the perfusion medium 24 h prior to the end of culture. Paraffin sections were prepared and BrdU epitopes were unmasked using microwave antigen retrieval.[13] Sections were permeabilized with 0.5% Triton X-100 solution for 20–30 min prior to labeling BrdU-positive nuclei with an antibody kit (Roche

Diagnostics, Labeling and Detection Kit I). All nuclei were counterstained with Hoechst 33258 (Molecular Probes) at 1 μg/ml. Coverslips were mounted using Fluormount-G (Southern Biotechnology Associates) for fluorescence microscopy.

Using transverse sections, BrdU-positive nuclei were quantified in the intimal, medial, and the inner adventitial artery layers that remained after initial artery preparation. Sections were imaged through a 10× objective using a DAPI filter for total nuclei and a FITC filter for BrdU-positive nuclei. Images were acquired for transverse sections at four equally spaced locations around the circumference. Total nuclei and BrdU-positive nuclei were counted using Image Pro Plus software (Media Cybernetics), and the average ratio of labeled nuclei to total nuclei was computed for each arterial sample.

Nonviable cells within the artery wall were detected using the membrane-impermeant dye ethidium homodimer-1 (Molecular Probes). Labeling was done after the arteries were harvested from organ culture using a protocol similar to that of Merrilees *et al.*[31] Briefly, short segments (10 mm) taken from 7-day cultured arteries were incubated at 37°C in static culture medium supplemented with 10% calf serum and ethidium homodimer (5 μM) for 24 h. Paraffin sections of the labeled tissue were prepared, deparaffinized, and incubated in 1 μg/ml Hoechst 33258 in PBS for 30 min at 37°C to counterstain all nuclei. Coverslips were mounted with Fluormount-G for observation under fluorescence microscopy. The labeling of nonviable cells would have ideally been done during the last 24 h of perfusion culture, but the required amount of ethidium would have been prohibitive.

Ethidium-labeled nuclei could only be distinguished from unlabeled nuclei in the medial layer. Sections were imaged through a 10× objective using a DAPI filter for total nuclei and a TRITC filter for ethidium-positive nuclei. The quantification of labeled nuclei in the medial layer was similar to that for BrdU-positive nuclei described above.

Statistics

Results are represented as mean ± SEM (standard error of the mean) unless otherwise noted. Paired Student's *t* tests were used to determine statistical significance between means, and *p* values less than 0.05 were considered significant.

RESULTS

Arterial Elongation

Figure 2 shows the variation in axial stretch ratio with time for arteries subjected to various axial loads. Arteries cultured at constant stresses of 500 and 1000 kPa continuously lengthened until rupture at 5.5 and 4 days, respectively. Arteries in Protocols 1 and 2 were continuously lengthened for 7 days without rupture. Arteries in Protocol 1 reached a stretch ratio of approximately 2 within the first day of culture and then asymptotically approached a maximum stretch ratio at an average rate of 2.0×10^{-7} s^{-1} over the remaining time in culture. After 7 days in culture, arteries in Protocol 1 reached an average stretch ratio of 2.13 ± 0.02 ($n = 8$), a 42% increase over the *in vivo* length.

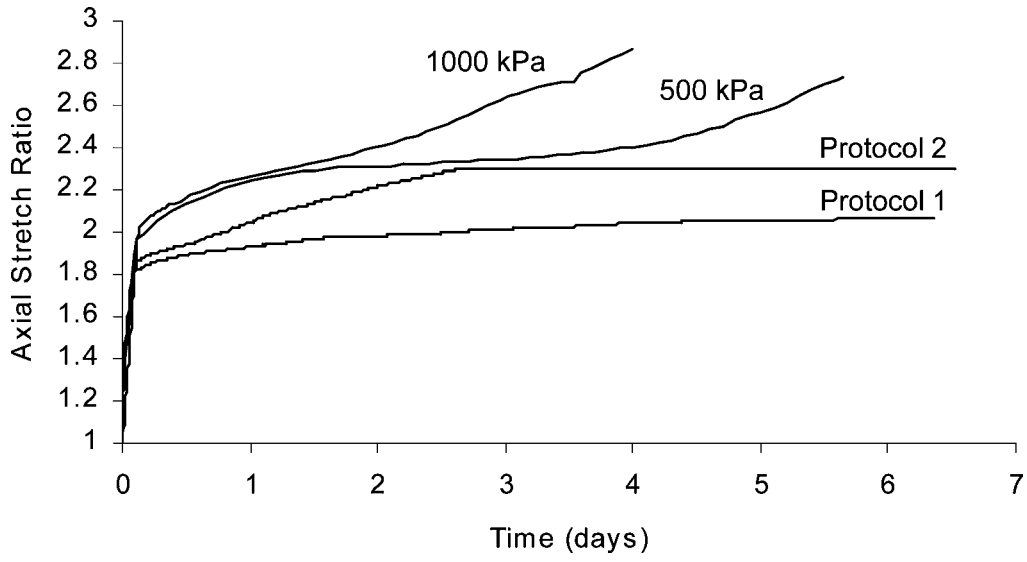

FIGURE 2. Typical creep behaviors of arteries cultured under sustained loading conditions. Arteries stretched in Protocol 1 asymptotically approached a maximum length whereas those stretched at 500 and 1000 kPa ruptured after 5.5 and 4 days, respectively. The creep behavior of arteries stretched in Protocol 2 was typically intermediate between that of arteries in Protocol 1 and the 500-kPa case. Each curve is a representative case of one artery within each group.

The length versus time curves for arteries stretched in Protocol 2 were intermediate between those of arteries stretched in Protocol 1 (250 kPa) and 500 kPa. Once the maximum stretch ratio was reached in Protocol 2, length was held constant and stress relaxation occurred. The axial stress in the stretched arteries of Protocol 2 decreased by an average of 62% during the period of constant length. Arteries in Protocol 2 reached an average stretch ratio of 2.36 ± 0.04 ($n = 9$), a 57% increase over the *in vivo* length.

The pre- and postculture no-load lengths of the arteries were compared to determine whether permanent length change occurred. Control arteries in Protocol 1 decreased in length by an average of 1.7 ± 0.8% and stretched arteries in Protocol 1 decreased in length by 0.9 ± 2.0%. Control arteries in Protocol 2 decreased in length by an average of 4.4 ± 0.9%, whereas stretched arteries in Protocol 2 increased significantly in length by 13 ± 7% on average.

Figure 3 plots permanent length change against the maximum axial stretch ratio in culture with each data point representing a single artery. A negative value indicates that final unloaded length was less than initial unloaded length. It is seen that permanent length increase only occurred in arteries that were stretched above a threshold value. A linear fit to the length data from stretched arteries resulted in an R^2 value of 0.89, indicating a strong linear correlation between permanent length increase and the stretch ratio in culture. The intersection of this linear fit with the horizontal axis occurred at a stretch ratio of 2.14. Above this threshold value, permanent arterial length increase was directly proportional to the maximum stretch ratio in culture. Below this threshold value, there was little permanent change in arterial length. Rupture was likely for arteries extended beyond a stretch ratio of approximately 2.4, and therefore the data to the right in Fig. 3 represent an approximate upper bound for stretch. The maximum axial stretch ratio achieved in culture was 2.57 and the maximum permanent length increase was 23%.

Smooth Muscle Viability

Figure 4 shows the changes in outer diameter (as absolute values) of the arteries in response to NE and SNP stimulation. Arteries in all groups had strong vasomotor responses to NE and SNP. None of the differences between control and stretched arteries was significant.

Cell necrosis was used as an index of arterial viability after culture. Figure 5 shows that in Protocol 1, there was no significant difference in the percentage of nonviable cells between control and stretched arteries. In Protocol 2, however, there was a significantly higher percentage of nonviable cells in the stretched arteries than in the controls ($p < 0.03$, $n = 9$). To further examine this difference, the nuclei in these arteries were classified as apoptotic if they were broken into clusters of small nuclear bodies or necrotic if the nuclei were intact.[34] Interestingly, there was no difference in the percentage of apoptotic nuclei between groups, but there were three times more necrotic nuclei in the stretched arteries than in the control arteries ($p < 0.02$, $n = 9$). Therefore axial stretch in Protocol 2 selectively increased necrotic cell death. However, the overall percentages of nonviable cells in arteries from *both* stretching protocols ranged between 0.4 and 1.5%, low values that were comparable to those measured in freshly harvested arteries using the same technique to label nonviable cells.

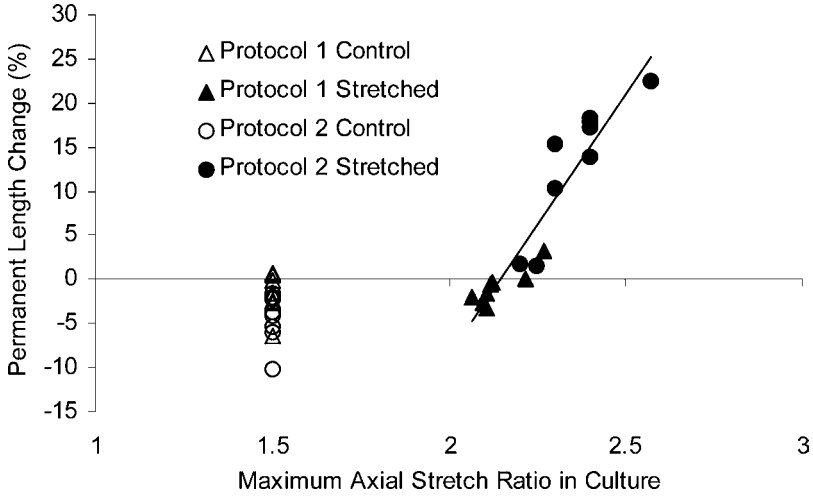

FIGURE 3. Permanent arterial length change (final unloaded length divided by initial unloaded length) is compared with maximum axial stretch ratio in culture (loaded length divided by initial unloaded length). Above the threshold stretch ratio of 2.14, permanent arterial length increase was directly proportional to the maximum stretch ratio in culture. Below this value, there was little change in arterial length. (Protocol 1 Stretched, $n = 8$; Protocol 1 Control, $n = 8$; Protocol 2 Stretched, $n = 9$; Protocol 2 Control, $n = 9$.)

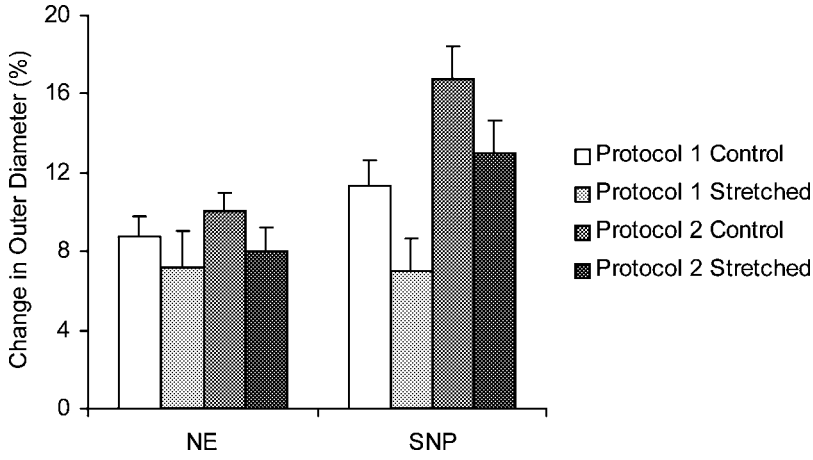

FIGURE 4. Average vasomotor response on day 7 from arteries cultured for 7 days. Arteries in all groups had strong vasomotor responses to norepinephrine (NE) and sodium nitroprusside (SNP). None of the differences between control and stretched arteries were significant. Results are plotted as absolute values. (Protocol 1, $n = 7$ pairs; Protocol 2, $n = 9$ pairs.)

Proliferation

Proliferation was much greater in the intimal and adventitial layers of the cultured arteries than in the medial layer. Figure 6 summarizes cell proliferation results in these layers of the arterial wall for both protocols. Cell proliferation was generally higher in the stretched arteries but only proliferation in the intima of stretched arteries in Protocol 1 was significantly greater than controls ($p < 0.05$, $n = 8$).

The results in Fig. 6 only represent proliferation during the last 24 h of culture. To estimate the accumulated effects of proliferation in the medial layer during the entire 7 days of culture, the smooth muscle population in the medial layer was measured before and after culture using 5-μm thick transverse artery sections. The total smooth muscle population per artery was calculated by multiplying the average number of medial nuclei per transverse section by the estimated total number of sections in the entire length of the artery. In Protocol 1, there was no significant difference in the smooth muscle population between stretched and control arteries. In Protocol 2, however, the number of smooth muscle nuclei decreased by $2 \pm 3\%$ in control arteries during culture but significantly increased by $20 \pm 7\%$ in stretched arteries ($p < 0.03$, $n = 9$).

Axial Modulus

Figure 7 shows a typical set of axial stress–strain curves acquired at a transmural pressure of 0 mmHg for a pair of arteries cultured for 7 days in Protocol 2. The pre- and post-culture curves of the control artery were similar. However, the stretched artery was more compliant after culture.

Table 1 summarizes the average axial moduli of cultured arteries at $\lambda_z = 1.5$ and 1.65 before and after culture for both protocols. Axial mechanical data were not available for stretched arteries in Protocol 2 at a stretch ratio of 1.5 and a pressure of 100 mmHg because many of these arteries had been lengthened and therefore buckled at this condition

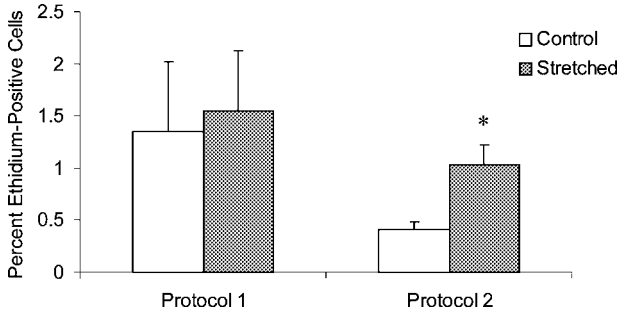

FIGURE 5. Nonviable cells detected by ethidium homodimer incorporation in the medial layer in arteries cultured for 7 days. * $p < 0.03$, Stretched vs. Control. (Protocol 1, $n = 8$ pairs; Protocol 2, $n = 9$ pairs).

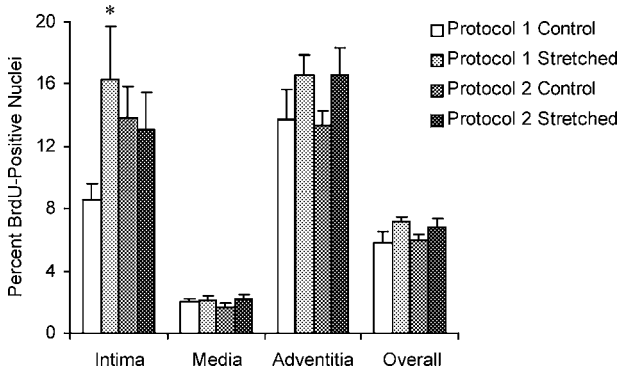

FIGURE 6. Average percentage of proliferating nuclei detected by BrdU incorporation during day 7 in arteries cultured for 7 days. * $p < 0.05$, Stretched vs. Control in Protocol 1. (Protocol 1, $n = 8$ pairs; Protocol 2, $n = 9$ pairs).

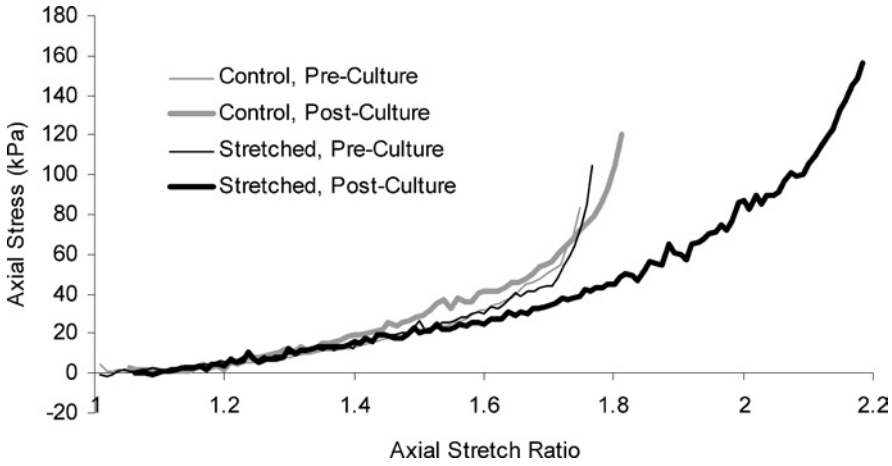

FIGURE 7. Typical set of axial stress–strain curves acquired at a transmural pressure of 0 mmHg from a pair of arteries cultured for 7 days in Protocol 2. The moduli of the stretched artery decreased during culture, and the artery could be stretched farther in the postculture test than it could in the preculture test.

after culture. The axial moduli of control arteries did not change significantly during culture for any of the conditions considered, but the axial moduli of arteries stretched using Protocol 2 decreased significantly during culture. The axial moduli for arteries stretched using Protocol 1 did not show any significant difference when compared to controls (Table 1).

Histology

Figure 8 compares the inner and outer wall morphologies of stretched and control arteries in transverse sections. Though some of the stretched arteries from Protocol 1 had intimal damage, the morphology of these arteries was generally very similar to that of controls as seen by comparing Fig. 8(A) with Fig. 8(B) and Fig. 8(D) with Fig. 8(E). In addition, examination of longitudinal sections showed that smooth muscle cell nuclei remained in the typical circumferential orientation after axial stretch.

In Protocol 2, focal structural damage was apparent in multiple layers of the stretched arteries, whereas the control arteries were undamaged. The internal elastic lamina (IEL) was intact in the sections from almost all control arteries [Fig. 8(A)]. In contrast, all the stretched arteries from Protocol 2 had at least one break in the IEL as shown in Fig. 8(C). The resulting gaps in the IEL ranged from approximately 5 to 90% of the lumen circumference. The remnants of the broken IEL were often still attached and curled out into the lumen. Beneath these breaks in the IEL, the nuclei of smooth muscle cells were larger and less intensely stained than in adjacent tissue below intact IEL and in control arteries. The smooth muscle cells also appeared disorganized beneath IEL breaks. An intact endothelial layer was present in most of the control arteries. In the elevated axial stress environment of Protocol 1, endothelial cells covered 50–75% of the lumen circumference of stretched arteries, whereas only 25–50% of the lumen circumference was covered in arteries stretched in Protocol 2.

TABLE 1. Axial moduli of arteries in Protocols 1 and 2 before and after culture.

			Axial modulus (kPa)			
			$\lambda_z = 1.5$		$\lambda_z = 1.65$	
Protocol	Artery group	Time point	0 mmHg	100 mmHg	0 mmHg	100 mmHg
1	Control	Before	253 ± 38	334 ± 57	410 ± 78	582 ± 134
		After	253 ± 14	304 ± 31	472 ± 23	740 ± 231
	Stretched	Before	269 ± 68	281 ± 89	549 ± 78	653 ± 316
		After	208 ± 28	274 ± 32	379 ± 112	534 ± 173
2	Control	Before	86 ± 9	104 ± 13	215 ± 82	258 ± 65
		After	89 ± 11	97 ± 10	138 ± 22	196 ± 9
	Stretched	Before	75 ± 6	88 ± 6	125 ± 15	170 ± 16
		After	50 ± 5*	—	88 ± 9*	105 ± 8*

Note. Values are mean ± SEM (Protocol 2, $n = 9$, Protocol 1, $n = 4$).
*$p < 0.02$ (after culture vs. before culture).

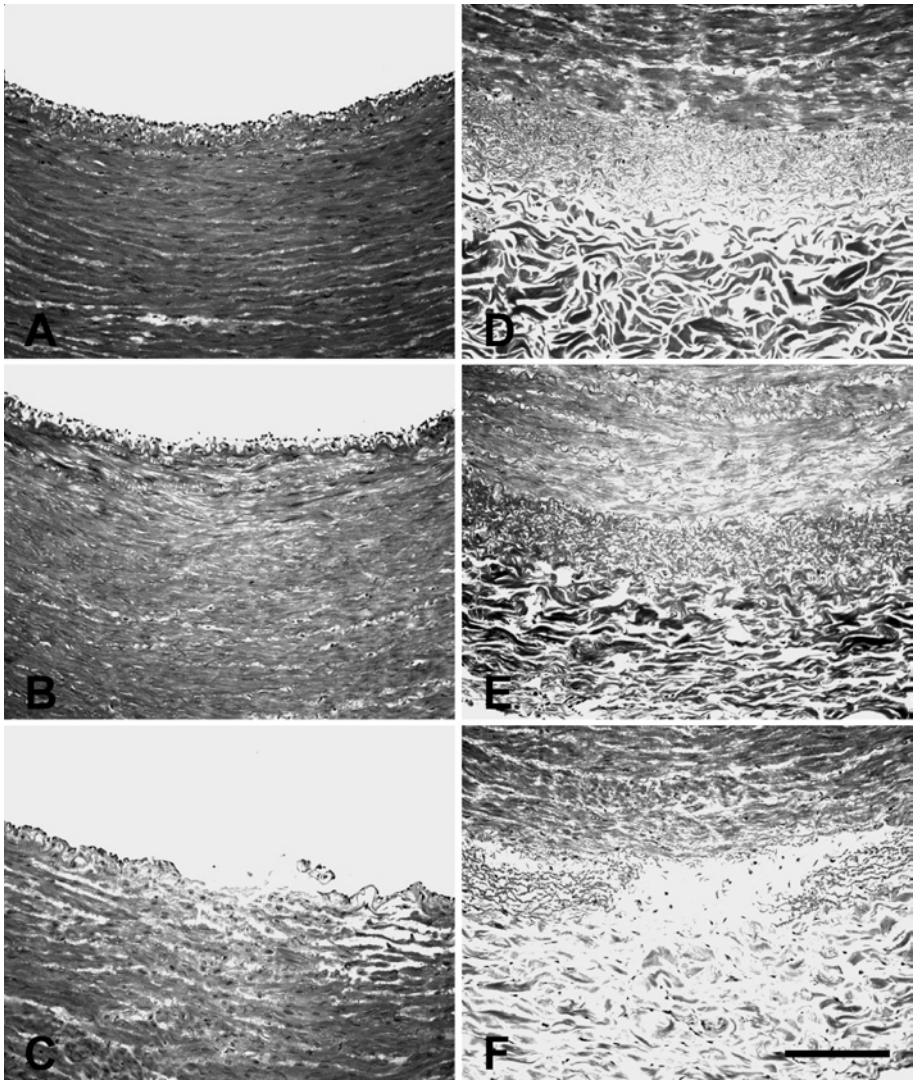

FIGURE 8. Comparison of the wall morphology in transverse sections from arteries cultured for 7 days in Protocol 1 and Protocol 2. Controls (A and D), Stretched in Protocol 1 (B and E), Stretched in Protocol 2 (C and F). Inner wall (A–C), outer wall (D–F). Note the focal damage to the internal elastic lamina (C), the medial layer (C and F), and the longitudinal fibers in the dense adventitial layer between the media and the loose adventitial collagen bundles (F). The lumen is at the top in each image. Hematoxylin and eosin. Scale bar is 200 μm.

Approximately 89% of the stretched arteries in Protocol 2 had disruptions to the longitudinal fibers in the dense adventitial layer that divides the medial layer from the loose collagen bundles in the adventitia [Fig. 8(F)], whereas none of the control arteries had any fiber damage [Fig. 8(D)]. In the stretched arteries, gaps in the dense adventitial layer where fibers appeared to be missing were up to 5% of the total circumference in length, and there were sometimes multiple gaps per cross-section. Medial tissue adjacent to breaks in the dense adventitial layer had morphology similar to that of medial tissue near breaks in the IEL.

All arteries that showed damage in the dense adventitial layer also had damage in the IEL, whereas some arteries only had damage to the IEL. These observations indicate that the IEL was more susceptible to damage from axial stretch than the dense adventitial layer.

DISCUSSION

In this study we have shown that arteries remain viable for 7 days in organ culture during the application of axial loads that approach but remain below the rupture limit. Permanent elongation was shown to begin when arteries reached a stretch ratio of 2.14 in culture, and up to 23% permanent elongation of the arteries was achieved using the protocols in this study.

Vasomotor responses induced by NE and SNP were similar to those reported in a previous study after lower intensity axial stimulation.[16] Also in agreement with that

report, the number of nonviable smooth muscle cells was below 2% in all artery groups tested. Although smooth muscle cell morphology was abnormal in focal areas of matrix damage in some stretched arteries, the overall results indicate that smooth muscle contractile function and viability are not sensitive to the range of axial stimulus intensity studied here.

The elevated strain environment was more detrimental to endothelial cells than to smooth muscle cells. Endothelial cells may have responded adversely to the higher strain in the IEL of stretched arteries. Though wall shear stress was maintained as close to 15 dyn/cm^2 as possible, shear stress may also have been high enough to cause damage to endothelial cells that became vulnerable due to the high strain. The damage to endothelial cells requires further study.

Though the smooth muscle cell proliferation rate during the last 24 h of culture in the medial layer was unaffected by elevated stretch, a higher rate of proliferation must have occurred at some point during the culture period for stretched arteries in Protocol 2. The 20% increase in the estimated smooth muscle cell population in the stretched group suggests that a transient proliferative response occurred during the 7-day culture period. Significantly elevated BrdU index was previously confirmed in arteries stretched 20% greater than their *in vivo* length for 5 days.[16] The rate of cell proliferation most likely peaked and returned to baseline levels before BrdU was added.[6] Correlating an increase in cell population with BrdU index is difficult, but a 20% increase in smooth muscle population is possible given a population doubling time on the order of days as reported for smooth muscle cells in culture.[5,37] It was hypothesized that a more intense axial stimulus would lead to a greater proliferative response. Clearly, the dose-dependency between elevated axial stretch and smooth muscle cell proliferation requires further investigation. *In vivo* experiments suggest a strong connection between axial stretch and smooth muscle cell proliferation.[24]

Both stress- and strain-controlled axial loads were achieved in the current organ culture system. In contrast, due to practical limitations, only strain-based protocols were reported in the literature regarding stretch of arteries *in vivo*. Arteries have been stretched *in situ* up to 140% greater than their *in vivo* (stretched) length using external fixators[11,18,23] and tissue expanders.[12,36] In this study, axial loading in Protocol 2 stretched arteries to 57% greater than their *in vivo* length on average. The smaller elongation observed here might be a result of the lower compliance of porcine carotid arteries compared to arteries investigated in the literature such as the rat femoral artery.[36]

Although permanent length change was not given in most reports in the literature, a 50% increase in unloaded length was reported for rat arteries after 15–20 min of axial stretch.[8] This value was considerably greater than the average 13% increase in unloaded length of the arteries stretched in Protocol 2. Our protocol applied a continuous axial load over several days as opposed to a sudden acute axial load. The elongations may therefore result from different mechanisms. Species and age differences may also account for the disparity between these two results.

Focal matrix damage appears to be a likely mechanism leading to increased arterial length in the unloaded state. However, except for periodic focal damage, arterial cross-sections were intact indicating that other mechanisms such as tissue avulsion,[24] matrix reorganization, or plastic deformation of matrix fibers in response to stretch are also important. A combination of these mechanisms probably produced the observed results, but the relative importance of each is unknown. Figure 3 suggests that matrix damage was initiated around a stretch ratio of 2.14 in the loaded condition. No signs of new matrix formation were observed in any arteries in this study,[9] suggesting that permanent elongation was mainly a passive process.

Structural damage to the IEL and the medial layer has been reported after arteries were aggressively stretched *in vivo*.[8,32] Little attention was given to the adventitia in these studies, but some of the histologic images showed evidence of adventitial damage after axial stretch.[32]

In this study, the most frequent type of damage in Protocol 2 was rupture of the IEL and the dense adventitial layer. The fact that broken fibers in the dense adventitial layer were always accompanied by IEL damage indicates that the IEL is more prone to damage under these conditions. This pattern may partially reflect the higher general state of stress in the IEL from combined transmural pressure and axial load. Damage of this nature was not seen in the previous study where the applied loads were smaller.[16]

Decreased axial mechanical moduli offered further evidence of matrix changes. Rupturing of matrix fibers would reduce the number of structural elements supporting the axial load, leading to more compliant axial behavior. Similarly, lower axial tensile strengths were reported for arteries immediately following axial stretch *in vivo*.[18] However, the reported return of tensile strength to control values after 16–24 weeks of recovery indicated active matrix repair after the stretching protocol ended. It is important to note that the maximum axial stresses that can be applied to arteries for 7 days without rupture were much lower than the average axial failure stress of 1080 ± 89 kPa found for these arteries in classic axial tensile tests performed on whole vessels.[9]

In contrast to the arteries stretched using Protocol 2, the arteries stretched using Protocol 1 did not experience significant changes in axial moduli following culture. This result was expected since these arteries did not experience significant changes in no-load length following culture. However, the general increase in axial modulus of arteries in Protocol 1 compared to those in Protocol 2 was unexpected. Several factors including animal-to-animal variability and reduced sample size may have contributed to this result.

Tearing of matrix fibers does not limit the extent of lengthening if new matrix formation concurrently reinforces the arterial wall. For example, it has been shown that bone-lengthening, which has been studied extensively, *only* occurs in the presence of matrix damage.[21,22] Alternately, if the rate of matrix deposition were similar to the rate of strain application, new matrix would presumably be deposited at a lower strain state than that of the original fibers under the applied load, thereby leading to a greater unloaded tissue length.[20] Indeed, significant lengthening of arteries *in vivo* has been reported with no evidence of damage.[24] The relative importance of focal injury and matrix reorganization as mechanisms leading to permanent lengthening in blood vessels requires further investigation.

There is a clinical demand for small-diameter vascular grafts. Autologous arteries are far superior to other grafts in these applications, and despite continuous progress, tissue engineered equivalents still face many challenges. Determining an appropriate axial stimulus to lengthen arteries could provide a means of generating autologous vascular graft tissue. Acutely stretched arteries have successfully repaired peripheral vascular defects in clinical situations that would have otherwise required interpositional vein grafts due to defect length.[35] In these cases, the highly stretched arteries remained patent 34 months after the procedures. The resilience of arteries to intense axial stimulation demonstrated in this study shows the potential to increase the supply of autologous small-diameter grafts by elongating existing arteries.

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