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CHARACTERIZATION OF CONTRACTILE PROPERTIES OF PORCINE MESENTERIC AND TRACHEOBRONCHIAL LYMPHATIC SMOOTH MUSCLE

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ABSTRACT

We performed morphometric and length-tension analyses comparing mesenteric and tracheobronchial lymph vessel segments to determine the potential of the latter tissue to regulate pulmonary and mediastinal lymph flow via alterations in smooth muscle tone. Fresh porcine lymph vessel rings were prepared for 1) in vitro *assessment of length-tension relationships and 2) histologic preparation and measurement of smooth muscle cross-sectional area (SMA). Mesenteric and tracheobronchial optimal vessel ring lengths were 3.1±0.2 and 3.5±0.2 mm, and maximum active tensions were 1518±25 and 1703±162 mg. Smooth muscle formed indistinct layers in each tissue, and only 30% of the smooth muscle was oriented circumferentially. Stress generated by the circular smooth muscle was similar to that generated by other types of vascular smooth muscle. In 75% of mesenteric vessel rings spontaneous contractions were observed that had a mean contraction frequency of 1.7±0.2 min^{-1} and a mean contraction amplitude of 349±35 mg, while only 40% of tracheobronchial vessels exhibited spontaneous contractions ($p<0.001$) that had a mean frequency of 0.6±0.2 min^{-1} ($p=0.0021$) and a mean contraction amplitude of 118±10 mg ($p<0.0001$). We conclude that tracheobronchial lymphatic vascular smooth muscle is capable of developing stress similar to that generated by mesenteric lymph vessels, and that spontaneous rhythmic contractile activity is qualitatively and quantitatively different in tracheobronchial than in mesenteric porcine lymph vessels. The data suggest that tracheobronchial lymph vessels are capable of regulating pulmonary and mediastinal lymph flow through intrinsic mechanisms. Such regulation may occur by alterations in vascular resistance rather than via spontaneous pumping activity.*

The importance of intrinsic mechanisms in the regulation of lymph flow is well recognized. These mechanisms are perhaps most critical in the mesenteric lymphatic vasculature, which relies primarily on rhythmic spontaneous contractions for the transport of fluid and nutrients. In some species, mesenteric lymphatic vessels contain well developed smooth muscle layers that are under the influence of neural (1) and humoral factors (2-4), and also have been shown recently to be modulated by the local release of endothelium-dependent factors (5,6) and by mechanical factors such as alterations in transmural pressure (7).

The contribution of intrinsic mechanisms to the regulation of flow in other regional lymph vessels, particularly the pulmonary and mediastinal lymphatic vasculature, is not as clearly defined. We have shown previously that tracheobronchial lymphatic vascular smooth muscle contractile responses are modified by humoral factors and by

endothelium-derived relaxing factor (8-10). Others have reported that some mediastinal lymph vessels are relatively fibrous, suggesting that their contribution to regulation of lymph flow through intrinsic mechanisms is small (11,12). In contrast, we have demonstrated that the active tensions developed by mesenteric and tracheobronchial lymphatic smooth muscle are similar, suggesting that some mediastinal lymphatic tissues play an active role in regulating lymph flow (13).

The present study was designed to explore further the capacity of tracheobronchial lymphatic smooth muscle to contribute to intrinsic regulation of lymph flow. Our previous experiments demonstrating the generation of similar active tensions in bovine mesenteric and tracheobronchial lymphatic smooth muscle suggested that the stress generated in these tissues was not of the same magnitude as that found in blood vessels. We performed morphometric analyses of porcine mesenteric and tracheobronchial lymph vessels, and determined that the generated stresses in these vessels were similar, and were of the same order of magnitude as those found in blood vessels. We also analyzed spontaneous rhythmic contractile activity, which was common in mesenteric vessel rings but occurred infrequently in tracheobronchial vessels. Our data demonstrate that porcine tracheobronchial lymph vessels have the capacity to modulate lymph flow through intrinsic mechanisms. This modulation may occur primarily through alterations in vascular resistance rather than through spontaneous pumping activity.

MATERIALS AND METHODS

Tissue Preparation

Blocks of mediastinal and mesenteric tissue from freshly slaughtered pigs (150 to 250 kg; male or female) were immersed in saline at 37°C. A 1% solution of Evans blue in saline was injected into lymph nodes in the mesentery and at the tracheobronchial junction, and the tissue blocks were incubated for 30 min at 37°C to permit staining of efferent lymphatic vessels. Lymph vessels measuring 2-5 mm diameter were ligated downstream and dissected sharply from surrounding tissue using microscissors under a binocular dissecting microscope.

Lymph vessels were cut into 5 mm width rings. Alternate rings were processed for either assessment of length-tension relationships or measurement of smooth muscle content. Rings used for study of length-tension relationships were suspended in a water-jacketed bath (10 ml) containing buffered Krebs solution (NaCl 118 mM; $NaHCO_3$ 24 mM; KCl 4.7 mM; KH_2PO_4 1.2 mM; $CaCl_2$ 1.6 mM; $MgCl_2$ 0.4 mM; dextrose 5.5 mM; pH 7.4) at 37°C continuously aerated with 95% O_2 and 5% CO_2. Each vessel ring was mounted onto two stainless steel wires. One wire was attached to a rigidly held glass rod within the bath. The other wire was fixed with a loop of 0000 braided silk ligature to a Grass FT.03 force-displacement transducer that was mounted on a rack and pinion to stretch the tissue preparation to desired lengths. At the conclusion of each experiment the length of the stretched vessel ring was measured with a micrometer. The rings were gently blotted and weighed (wet weight).

Determination of Length-Tension Relationships

For vessel rings mounted in organ baths, resting tension was set at 100 mg following a 1 hr equilibration period. Isometric contractions were produced by bathing the lymph vessel rings in 65 mM KCl-substituted Krebs perfusate following intermittent cumulative tissue ring length increases of 100μm. A 10 min equilibration period with the vessel rings in buffered Krebs elapsed following each length increase, at the conclusion of which a new resting tension was measured. Both tracheobronchial and mesenteric vessel rings exhibited stress relaxation in response to 100μm length increases, and the 10 min

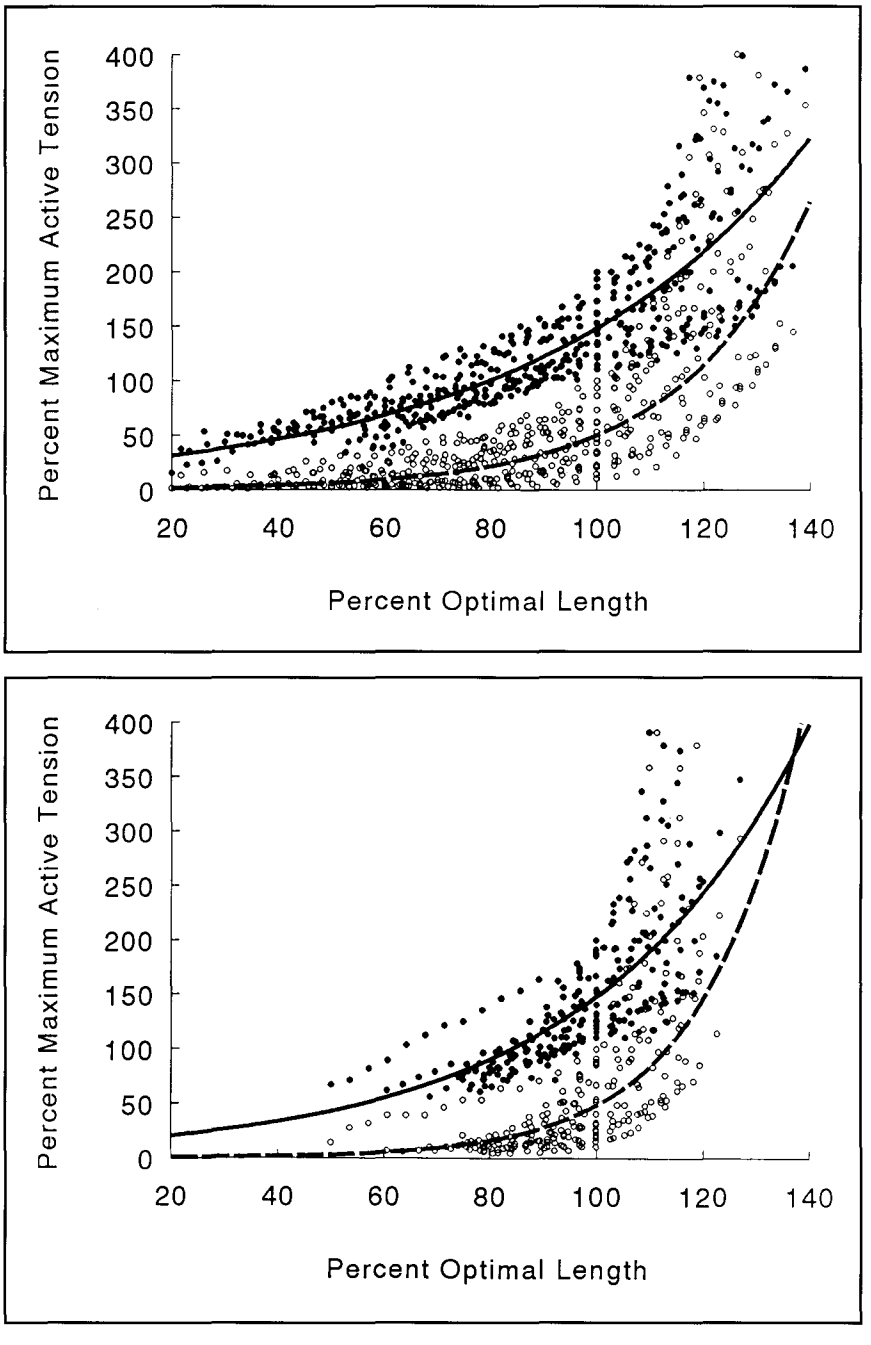

Fig. 1. Porcine mesenteric and tracheobronchial lymphatic vessel ring length-resting and length-total tension relation-ships. Data are expressed as percent maximum active tension (AT_{max}) versus percent optimal length (L_o). (Top): A total of 461 data points from 18 mesenteric lymph vessel rings are included for each curve. The curves describe exponentials expressed as $AT_{max}=0.79\ e^{0.04Lo}$ (resting tension, dashed line; r=0.82) and $AT_{max}=21.4\ e^{0.02Lo}$ (total tension, solid line; r=0.90). (Bottom): A total of 248 data points from 18 tracheobronchial vessel rings are included for each curve. The curves describe exponentials expressed as $AT_{max}=0.2\ e^{0.05Lo}$ (resting tension, dashed line; r=0.74) and $AT_{max}=12.54\ e^{0.02Lo}$ (total tension, solid line; r=0.80).

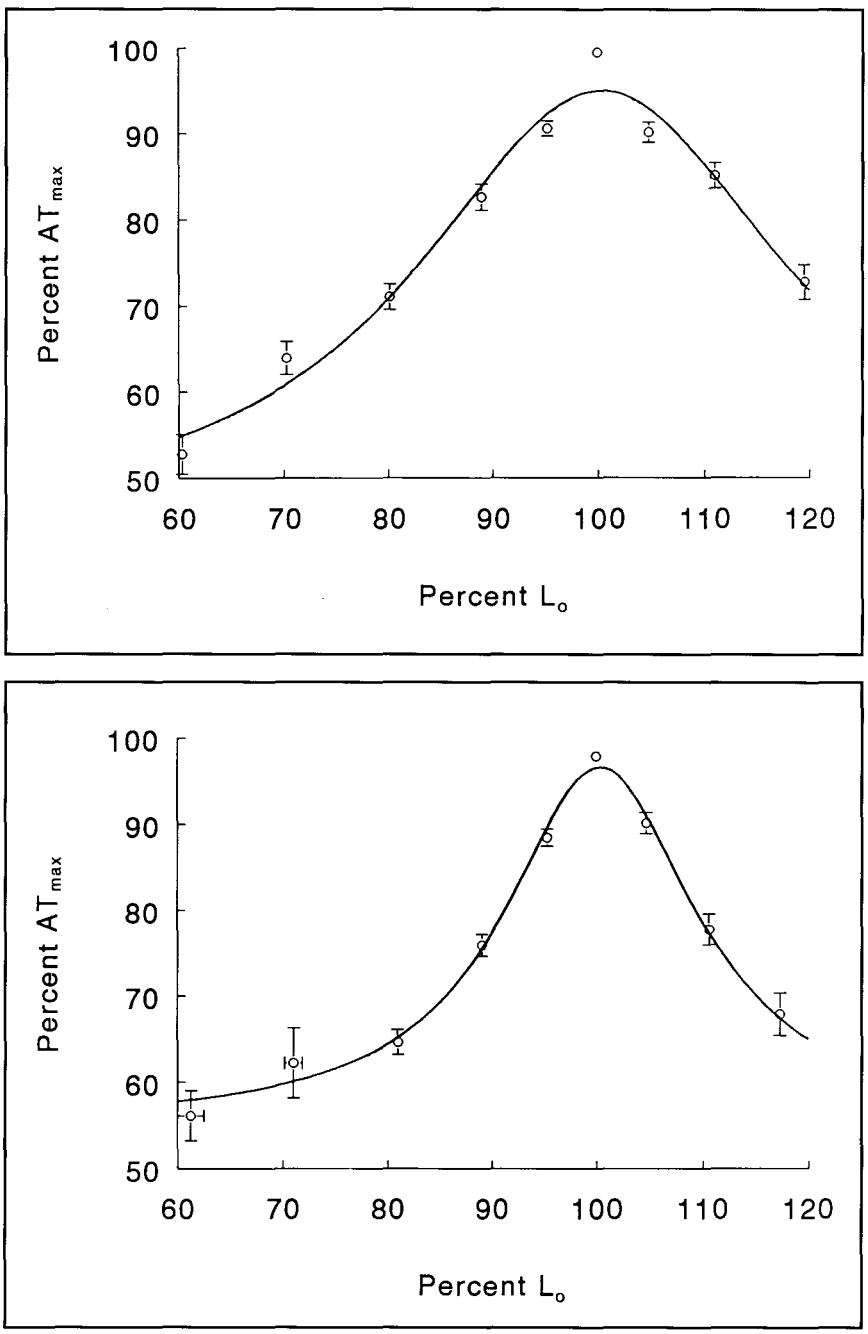

Fig. 2. Porcine mesenteric and tracheobronchial lymphatic vessel ring length-active tension relationships. Data are expressed as percent maximum active tension (AT_{max}) versus percent optimal length (L_o). (Top): A total of 461 data points from 18 mesenteric vessel rings are included. The curve is best described by a third order polynomial expressed as $AT_{max}=49.8-1.69L_o+ 0.04L_o^2-0.0002L_o^3$ (r=0.77). (Bottom): A total of 248 data points from 18 tracheobronchial vessel rings are included. The curve is best described by a third order polynomial expressed as $AT_{max}=402.7-15.3L_o+0.2IL_o^2-0.0009L_o^3$ (r=0.68).

equilibration period was found in preliminary experiments to permit vessel rings to reach a stable resting tension. In vessel rings exhibiting spontaneous contractions, resting tension was measured at the point of maximal relaxation between contractions. Bathing these vessel rings in 65 mM KCl resulted in sustained contractions that temporarily abolished phasic contractile activity, enabling assessment of total tension. Active tension was calculated as the difference between total tension in response to 65 mM KCl and the preceding resting tension. The vessel ring length (half-circumference) was determined using a micrometer at the conclusion of each experiment. Optimal vessel ring length (L_o) was calculated at the point where maximum active tension (AT_{max}) was generated, and the resting tension at this point was determined as the optimal resting tension. Vessel ring length was expressed as a percent of L_o, and active tension was expressed as a percent of AT_{max}. Vessel rings were excluded from analysis if optimal resting tension exceeded AT_{max}.

Measurement of Smooth Muscle Content

Vessel rings were fixed in 10% formalin and embedded in paraffin. Sections were taken transversely at 7μm and stained with hematoxylin and eosin. Computer assisted contour tracing was performed by projecting tissue images onto the plate of a Hewlett-Packard 9878 Digitizer using a Neo-Promar projecting microscope (Leitz; final magnification of 200X) as previously described (14,15). Profiles were traced of the internal margin of the vessel lumen, the interface between the smooth muscle and the adventitia, and the outer margin of the adventitia. Data were stored and analyzed by means of a Hewlett-Packard 9845A computer. Contour tracings were used to quantitate lumen area, total area, and smooth muscle area of each vessel ring cross section.

Calculation of Stress Generation

Vessel ring cross-sectional area was calculated as $A=W/L_o \rho$ where A=area in mm^2, W=vessel ring weight in mg, L_o=optimal vessel ring length in mm, and ρ was assumed to be 1.06 mg mm^{-2} (16). Using data from contour tracings of each paired histologic specimen, smooth muscle area was divided by total vessel ring tissue cross-sectional area to yield a fraction of vessel ring cross-sectional area made up of smooth muscle. This fraction was multiplied by the calculated cross-sectional area from each paired vessel ring, yielding a calculated cross-sectional area composed of smooth muscle. Stress (mN mm^{-2}) was determined by dividing AT_{max} by the calculated vessel ring smooth muscle area.

Assessment of Spontaneous Contractile Activity

Using a separate group of vessels, tracheobronchial and mesenteric vessel rings were mounted and equilibrated. Maximum active tension and optimal resting tension were determined as described above. The vessel rings were observed at optimal resting tension for 1 hr, and generated tensions were continuously recorded. At the conclusion of the observation period vessel ring length and weight were measured as described above. Tracings were analyzed for spontaneous contractions, which were defined as a discrete increase in tension of 50 mg or more followed by a return to baseline tension. Contractions were classified according to both rhythm and morphology. Rhythm was described as either regular or irregular, while morphology was classified as monomorphic or polymorphic. Spontaneous contractile activity was evaluated for frequency (contractions/min) and amplitude (maximum tension minus mean tension between contractions) as previously described (6).

Analysis of Data

Data are expressed as mean ± S.E.M. Differences between continuous variables were

examined using unpaired t-tests, and differences between discontinuous variables were compared using χ^2 analysis. Statistical significance was claimed when $p<0.05$. Curves for resting tension and total tension were fit using an exponential function, while curves for active tension were fit using a third order polynomial function.

RESULTS

Determination of Length-Tension Relationships

Twenty-eight mesenteric vessel rings from 9 animals were mounted, 10 of which were excluded from analysis due to inadequate AT_{max}. All had paired vessel rings that underwent histologic analysis. Mean mesenteric vessel ring weight was 6.05±0.59 mg. Twenty-four tracheobronchial vessel rings from 4 animals were mounted, 6 of which were excluded from analysis due to inadequate AT_{max}. All had paired vessel rings that underwent histologic analysis. Mean tracheobronchial vessel ring weight was 6.66±0.45 mg.

Changes in resting tension and total tension in response to KCl following cumulative increases in vessel ring length produced non-linear curves characteristic of biologic tissues *(Fig. 1)*. Mean resting tension at L_0 was 576±75 mg for mesenteric vessel rings and 807±115 mg for tracheobronchial vessel rings (p=N.S.). Optimal resting tension was 48.8±0.6% of AT_{max} for mesenteric vessels and 52.6±0.7% of AT_{max} for tracheobronchial vessels. Optimal vessel ring length averaged 3.1±0.2 mm and 3.5±0.2 mm for mesenteric and tracheobronchial vessel rings, respectively.

The relationship between active tension and vessel ring length demonstrated a gradual ascending limb, a broad peak at L_0, and a steeper descending limb as L_0 was exceeded *(Fig. 2)*. Slopes of the ascending and descending limbs were steeper in tracheobronchial vessel rings than in mesenteric vessel rings. For mesenteric vessel rings, AT_{max} was 1518±255 mg, while for tracheobronchial vessel rings it was 1703±162 mg.

Measurement of Smooth Muscle Content

Contour tracing of the lumen and outer tissue margins was straightforward. The outer margin of the smooth muscle layer was moderately well defined in most specimens *(Fig. 3)* but, in general, was better defined in mesenteric vessel rings than in tracheobronchial vessel rings. Although components of inner circular and outer longitudinal smooth muscle layers were identified in all vessels, it was not possible to distinguish reliably between these layers for purposes of contour tracing. We estimated that the inner circular layer made up one-third of the thickness of the entire smooth muscle layer, but this value was not quantitative and was not used in our analyses.

The average total vessel cross-sectional areas were 1.51±0.44 mm^2 and 1.80±0.19 mm^2 for mesenteric and tracheobronchial vessel rings, respectively. In mesenteric vessels, mean smooth muscle area was 0.34±0.04 mm^2 and mean lumen area was 0.29±0.07 mm^2. In tracheobronchial vessels, mean smooth muscle area was 0.67±0.11 mm^2 ($p<0.01$ versus mesenteric vessel rings) and mean lumen area was 0.14±0.02 mm^2 (p=N.S. versus mesenteric vessel rings).

Calculation of Stress Generation

Vessel ring cross-sectional areas calculated on the basis of vessel ring weight and L_0 were 1.95±0.23 mm^2 and 1.86±0.11 mm^2 for mesenteric and tracheobronchial vessel rings, respectively. For mesenteric vessel rings, smooth muscle area was 28.8±2.4% of the total vessel ring area by the contour method, yielding a calculated smooth muscle area of 0.52±0.06 mm^2. Generated stress was calculated at 35.8±8.2 mN mm^{-2}. For tracheobronchial vessel rings, smooth muscle area was 36.9±3.3% of the total vessel ring

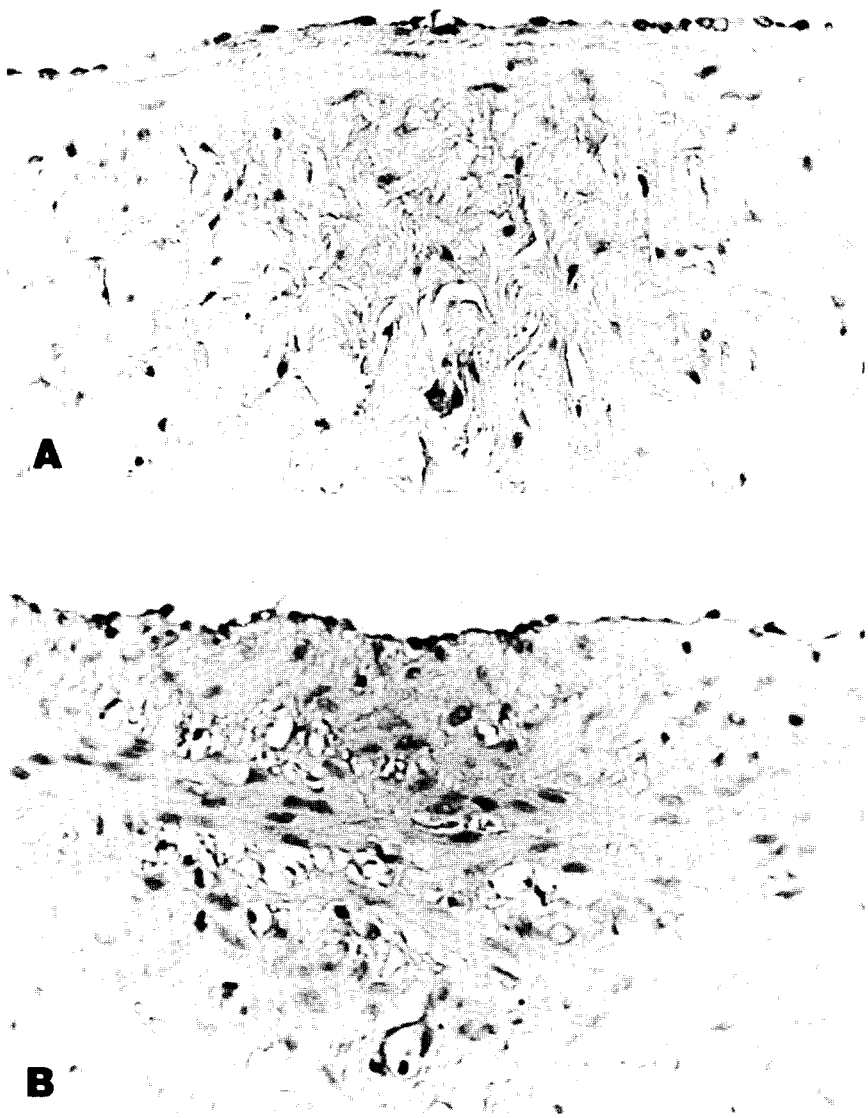

Fig. 3. Histology of porcine lymphatic vasculature, demonstrating a well-developed media in both tracheobronchial (A) and mesenteric (B) vessels. Orientation of the smooth muscle is not clearly demarcated into circumferential and longitudinal layers in either vessel type. The media in tracheobronchial vessels contained more connective tissue than was evident in mesenteric vessels. (Hematoxylin and eosin; original magnification x425.)

area by the contour method (p=0.055 versus mesenteric vessel rings), yielding a calculated smooth muscle area of 0.70±0.09 mm^2. Generated stress was calculated as 31.2±5.2 $mN\ mm^{-2}$.

Assessment of Spontaneous Contractile Activity

Sixty-five mesenteric vessel rings from 9 animals were mounted, in which the mean vessel ring weight was 8.5±0.6 mg and the average optimal vessel ring length was 2.7±0.1 mm. The mean maximum active tension was 1631±128 mg, and the average optimal resting tension was 677±28 mg. Forty-seven tracheobronchial vessel rings from 6 animals

were mounted, in which the mean vessel ring weight was 10.3±0.6 mg and the average optimal vessel ring length was 4.9±0.2 mm. The mean maximum active tension was 1770±199 mg, and the average optimal resting tension was 755±21mg.

Forty-nine of 65 (75%) mesenteric vessel rings exhibited spontaneous contractions which were characterized by a regular rhythm and monomorphic waveforms in 24, either regular rhythm or monomorphic waveforms in 13, and irregular rhythm and polymorphic waveforms in 11 *(Fig. 4)*. The mean contraction frequency in vessel rings exhibiting contractions was 1.7±0.2 min^{-1} and the mean contraction amplitude was 349±35 mg. In contrast, only 19 of 47 (40%) tracheobronchial vessel rings demonstrated spontaneous contractions ($p<0.001$ versus mesenteric vessel rings). The contractions were qualitatively different than those recorded in mesenteric vessel rings, characterized by a regular rhythm and monomorphic waveforms in 3, either a regular rhythm or monomorphic waveforms in 3, and an irregular rhythm and polymorphic waveforms in 14 ($p<0.01$). The mean contraction frequency in those vessels exhibiting spontaneous contractions was 0.6±0.2 min^{-1} and the mean contraction amplitude was 118±10 mg ($p=0.0021$ and $p<0.0001$ versus mesenteric vessel rings).

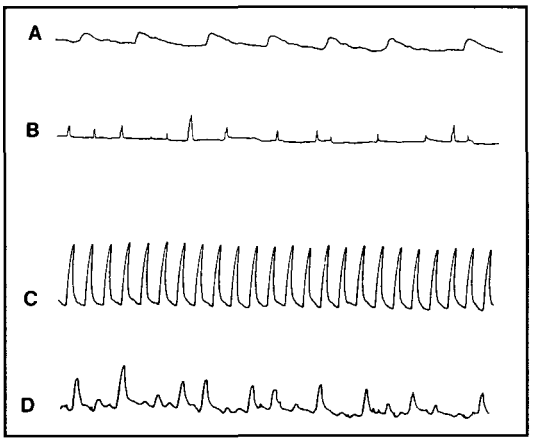

Fig. 4. Representative polygraph tracings of contractile activity in porcine lymph vessel rings. Some tracheobronchial vessel rings exhibited spontaneous, low amplitude contractions that had regular rhythm and monomorphic waveforms (A), while in the majority of vessel rings with spontaneous contractions the waveforms were low in amplitude, irregular in rhythm, and polymorphic (B). Most mesenteric vessel rings exhibited high amplitude contractions that had regular rhythm and monomorphic waveforms (C), while in the remaining vessel rings with spontaneous contractions the waveforms were of variable amplitude, rhythm, and morphology (D).

DISCUSSION

A primary function of the lymphatics, the collection and transport of interstitial fluid, is perhaps nowhere as important as in the lung. Mediastinal lymphatics have a potential role in regulating lung water, and their function may be the rate-limiting factor in the clearance of pulmonary edema (17-19). Modulation of lymph flow in the thorax is due, in part, to extrinsic forces such as negative intrapleural pressure, respiratory motions of the diaphragm and lungs, and the beating heart.

It is becoming increasingly evident that intrinsic factors contribute substantially to the regulation of lymph flow. This mechanism is of primary importance in the mesenteric lymph vessels, which exhibit spontaneous contractions of well-defined circumferentially-oriented smooth muscle in most species that have been studied (20). Whether spontaneous alterations in smooth muscle tone in mediastinal lymphatics contribute to the regulation of lung water is not known. Previous reports have shown that some mediastinal lymph vessels contain relatively more fibrous tissue and less smooth muscle than mesenteric lymphatics (11,12), and thus may not have the capacity for modulation of lymph flow through intrinsic mechanisms. In contrast, our previous findings demonstrated that active tension and generated stress were similar in bovine mesenteric and tracheobronchial tissues, suggesting that some mediastinal lymphatic vessels are capable of important regulation of tone (13).

The present study was performed to define further the capacity of tracheobronchial lymph vessels to modulate lymph flow. Although our previous work demonstrated that stresses generated by mesenteric and tracheobronchial lymph vessel rings were similar, these stresses were not comparable to those reported for other vascular tissues. Therefore, we performed morphometric analyses comparing mesenteric and tracheobronchial lymph vessels to permit a more accurate comparison between lymph vessels and blood vessels. In addition, spontaneous contractile activity has not been characterized in tracheobronchial lymph vessels in any species, although it is an important means of lymph transport. Finally, the use of porcine tissue for the current study necessitated standardization of this new model and allowed comparison of results with those previously reported for bovine tracheobronchial and mesenteric lymph vessels.

We found that maximum active tension and optimal resting tension in porcine tracheobronchial and mesenteric lymph vessel rings were similar and were of the same order of magnitude as those previously described in bovine lymph vessel rings (13). The length-tension curve for tracheobronchial vessel rings had a less pronounced plateau than did the curve for mesenteric vessel rings, suggesting that the latter tissue possesses relatively greater compliance or reduced tissue elastic modulus. These findings are similar to those described in bovine mesenteric and tracheobronchial lymphatic vascular tissue.

Although the optimal vessel ring lengths and weights in porcine tracheobronchial and mesenteric vessels were not significantly different, the measured smooth muscle area was greater in tracheobronchial than in mesenteric lymph vessel rings. When measured smooth muscle area was corrected for relaxation and shrinkage during fixation, however, the resultant calculated smooth muscle areas for the two tissue types were remarkably similar and yielded similar calculated stresses.

The histologic appearance of the porcine lymph vessels contrasted to that of some other species, in which well-defined circumferential and longitudinal layers are often described. In most vessel segments studied we were able to identify a well developed media that had discrete outer margins, but found that the orientation of smooth muscle cells within the media could not be clearly separated into longitudinal and circumferential layers. As a result, we were only able to estimate that about 30% of cells were oriented circumferentially. The impact of this organization on intrinsic function of porcine lymph vessels is not clear.

We estimated that, since the circumferentially oriented smooth muscle in porcine lymph vessels is only one-third of the total smooth muscle cross-sectional area, stress generated by this smooth muscle is three-fold greater than what was actually measured, or about 100 mN mm^{-2}. This value is of the same order of magnitude as generated stresses calculated for other smooth muscles, including visceral (87 mN mm^{-2}) (21), airway (108 mN mm^{-2}), (22), and arterial media only (72-222 mN mm^{-2}) (23,24). The results are not unexpected, as one would predict that smooth muscle contractile properties are similar regardless of the organ in which they reside.

Spontaneous rhythmic contractile activity was common in porcine mesenteric vessel rings, as is characteristic of mesenteric lymph vessels in other species. This activity is one component of the intrinsic lymph pump, which is primarily responsible for mesenteric lymph transport. In contrast, the spontaneous contractions observed in tracheobronchial vessel rings were less frequent, had lower amplitude, and were morphologically different. This suggests the possibility that, if intrinsic regulation of flow occurs in this tissue, it may do so through mechanisms other than an intrinsic lymph pump. We suggest that our data are consistent with the regulation of mediastinal lymph flow through alterations in vessel diameter, but that these alterations may serve to modulate resistance

to lymph flow rather than as a means to pump lymph.

Our data provide one of the first anatomic and functional descriptions of porcine mesenteric and tracheobronchial lymph vessels and demonstrate that activity in these tissues can be reproducibly measured. The findings corroborate results from our previous study using bovine tissue, which showed that active tension and generated stress are similar in mesenteric and tracheobronchial lymphatic vascular smooth muscle. The current data suggest that tracheobronchial lymph vessels have the capacity to modulate flow of lymph within the mediastinum, but that they may act as resistance vessels rather than as active lymph pumps. Additional investigations of tracheobronchial lymph vessels using whole mounted segments or *in vivo* analysis will be necessary to define further their functional characteristics.

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