Transmural pressure in rat initial subpleural lymphatics during spontaneous or mechanical ventilation

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Moriondo, Andrea, Sylvain Mukenge, and Daniela Negrini. Transmural pressure in rat initial subpleural lymphatics during spontaneous or mechanical ventilation. Am J Physiol Heart Circ Physiol 289: H263–H269, 2005. First published April 15, 2005; doi:10.1152/ajpheart.00060.2005.—The role played by the mechanical tissue stress in supporting lymph formation and propulsion in thoracic tissues was studied in deeply anesthetized rats (n = 13) during spontaneous breathing or mechanical ventilation. After arterial and venous cannulation and insertion of an intratracheal cannula, fluorescent dextran were injected intrapleurally to serve as lymphatic markers. After 2 h, the fluorescent intercostal lymphatics were identified, and the hydraulic pressure in lymphatic vessels (Plymph) and adjacent interstitial space (Pint) was measured using micropuncture. During spontaneous breathing, end-expiratory Pint and corresponding Plymph (and adjacent interstitial space) were −2.5 ± 1.1 (SE) and 3.1 ± 0.7 mmHg (P < 0.01), which dropped to −21.1 ± 1.3 and −12.2 ± 1.3 mmHg, respectively, at end inspiration. During mechanical ventilation with air at zero end-expiratory alveolar pressure, Plymph and Pint were essentially unchanged at end expiration, but, at variance with spontaneous breathing, they increased at end inspiration to 28.1 ± 7.9 and 28.2 ± 6.3 mmHg, respectively. The hydraulic transmural pressure gradient (∆Pint = Plymph − Pint) was in favor of lymph formation throughout the whole respiratory cycle (∆Pint = −6.8 ± 1.2 mmHg) during spontaneous breathing but not during mechanical ventilation (∆Pint = −1.1 ± 1.8 mmHg). Therefore, data suggest that the intercostal lymphatics drain the thoracic tissues during spontaneous breathing or passive lung inflation. The role of the tensile and/or compressive tissue stresses applied during active or passive chest expansion was evaluated on the basis of the net pressure gradients developing during the entire respiratory cycle across the lymphatic vessel wall.

MATERIALS AND METHODS

The experiments were performed on 13 adult male rats [body weight 317 ± 10 (SE) g] in accordance with the guidelines of the Animal Care and Use Committee of the Ministry of University and Research. Rats were deeply anesthetized with 2.5 ml/kg body wt of an anesthetic cocktail of saline solution containing 0.25 g/ml urethane plus 10 mg/ml pentobarbital sodium and 0.03 mg/ml fentanyl. Subsequently, boluses of 0.1 ml of the anesthetic cocktail were given intravenously throughout the experiment to maintain deep and stable anesthesia, the level of which was assessed on the basis of the disappearance of the corneal reflexes. Once anesthetized, the animals were turned supine on a warmed (37°C) blanket; they were tracheotomized and left to breathe spontaneously through an intratracheal cannula.

Blunted-tipped saline-filled plastic catheters were inserted into a carotid artery and a jugular vein and were connected to physiological pressure transducers (model P23 XL; Gould Electronics). In addition, to measure esophageal pressure, a saline-filled PE50 catheter was advanced in the esophagus with its blunt tip positioned in correspondence with the second third of the sternum.

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Systemic arterial, venous, and esophageal pressures were monitored throughout the whole experiment by conveying the pressure signal to an amplifier and a signal conditioner (model 6600; Gould Electronics). The signal was then digitized with an analog-to-digital board and displayed on the monitor screen using dedicated LabView software (National Instruments, Austin, TX).

A heated pneumotachograph (model 8420; Hans Rudolph) equipped with a dedicated pneumotach amplifier (model 1110A; Hans Rudolph) was connected to the tracheal cannula for continuous recording of respiratory flow. Tidal volume was obtained from integration of the respiratory flow signal performed with the LabView software.

Intravital labeling of intercostal pleural lymphatic network. The skin and the external intercostal muscles on the right side of the thorax were cleared to expose the ribs and the internal intercostal muscles. A stainless steel cannula (external diameter 0.7 mm; internal diameter 0.4 mm) connected to a plastic catheter and to a three-way stopcock was filled with saline solution containing 20% fluorescent dextrans (FITC; molecular weight 70,000; Sigma, St. Louis, MO). The blunted cannula tip was tangentially inserted into the pleural space while saline was flushed over the insertion point to prevent air entrance into the pleural space, and the cannula was then gently advanced along the lung surface. Subsequently, 0.5 ml of the fluorescent solution was injected intrapleurally, the cannula was withdrawn, and the animal was turned to right lateral decubitus position.

Two hours after FITC injection, the animal was turned back to supine position. The internal intercostal muscle fibers of the parasternal region in the third to seventh intercostal spaces were carefully removed to expose the lymphatic vessels containing the fluorescent dye and were thus clearly detectable though a stereomicroscope (magnification ×60–100; Zeiss SV 11) when illuminated with a fluorescent mercury lamp (light wavelength 425/65 nm, HBO 50 W; Zeiss) as in the example of Fig. 1. The microscope image was captured with a digital video camera (Axiocam; Zeiss), processed with a dedicated board, and displayed on a color monitor with a maximal magnification on the video screen of ×200. Digital images on the screen were stored and successively automatically analyzed for vessel dimensions with the use of image analysis software (KS300; Zeiss).

The diameter of the visualized and micropunctured lymph vessels computed on the stereomicroscopic images ranged between ~50 and ~300 μm.

The arrangement of the intercostal lymphatics exposed and micropunctured in the present study was very variable. Some vessels were located either in the paravertebral loose interstitial tissue or in the endothoracic fascia of the intercostal space, running parallel to the longitudinal axis of the sternum; others followed instead the cranial curvature of the ribs, describing an angle of 60–90° with respect to the sternum axis. Most vessels were located deeply in the subpleural layer that is very thin and strictly connected to the parietal mesothelium. Therefore, the surgical approach required a careful removal of both the external and the internal intercostal muscles down to the endothoracic fascia. Given the size of the rat intercostal spaces, such a preparation could usually be satisfactorily performed only in the third to seventh intercostal spaces, whose ventral width is larger compared with the other spaces in supine rats. Hence, \( P_{\text{lymph}} \) and \( P_{\text{int}} \) were recorded only in the ventral portion of supine animals at the same or slightly higher level of the right atrium.

Recording of intraluminal lymphatic and interstitial hydraulic pressure. The hydraulic pressures within the visualized lymphatic vessel lumen (\( P_{\text{lymph}} \)) and in the adjacent intercostal interstitial space (\( P_{\text{int}} \)) were measured by means of the micropuncture technique while the animal was spontaneously breathing though the intratracheal cannula. Pressure recordings were performed using glass micropipettes, with a taper of ~150 μm beveled down to a tip diameter of 2–4 μm and filled with 1 M NaCl solution, that were inserted in a pipette holder filled with the same solution. The holder was connected to a mineral oil-filled pressure transducer (Gould Instruments System) motor driven by an electrohydraulic system (Dual Servonull pressure measuring system; Vista Electronics, Ramona, CA). The amplified pressure signal was conveyed and displayed on the monitor screen utilizing the same equipment described for the vascular pressure recordings. Before its use, each micropipette was calibrated in a modified Lucite box by imposing step changes of ±5 mmHg in the box chamber; pipettes displaying a nonlinear calibration in the pressure range ±30 mmHg were discarded. After calibration, pipette holders were mounted in two three-dimensional hydraulic micromag.
were obtained. After the pipette tip was positioned into the chosen inspiration for a tidal volume of 6 ml during which the animals were always spontaneously breathing. Electrical zeroing of the recording system was performed before and immediately after each measurement by dipping the micropipette tip in a saline pool positioned at the same height of the pipette insertion point. Criteria for acceptance of the micropipette pressure recordings were 1) an unchanged electrical zero of the system upon withdrawal from the tissue compared with preinsertion value, 2) a stable pressure reading for at least 2 min, and 3) repeated measurements from the same area within 1 mmHg of each other.

In the same animal, up to four paired \(P_{\text{lymph}}\) and \(P_{\text{int}}\) recordings were obtained. After the pipette tip was positioned into the chosen lymph vessel, another pipette was inserted in the adjacent interstitium \(\sim 10 \mu\text{m}\) from the outer lymphatic vessel surface. The microusurgical procedure to expose the lymphatics deep in the parasternal intercostal spaces required 30–60 min; hence, measurements of \(P_{\text{lymph}}\) and \(P_{\text{int}}\) began at \(\sim 150–180\) min after dextran injection and 180–210 min after anesthesia induction. On average, experiments lasted up to 6 h, during which the animals were always spontaneously breathing.

In a small group \((n = 3)\), after pressure recording during spontaneous breathing, rats were paralyzed with a single intravenous dose of pancuronium bromide \((\sim 8 \text{ mg/kg})\) and then passively ventilated (ventilator model 7025; Ugo Basile, Comerio, Italy) with room air, saline pool positioned at the same height of the pipette insertion point. immediately after each measurement by dipping the micropipette tip in a saline pool. The correlation existing between the end-inspiratory \((\Delta P_{\text{tm},i})\) and the corresponding end-expiratory \((\Delta P_{\text{tm},e})\) transmural pressure gradient was assessed by performing the Pearson product moment correlation with significance for \(P > 0.05\).

To appreciate the relative importance of cardiogenic and respiratory swings in determining the overall shape of the \(P_{\text{lymph}}\) and \(P_{\text{int}}\) traces, we performed a frequency-domain analysis of the time-domain pressure traces by means of fast Fourier transformation (FFT). Reference frequencies for cardiac and respiratory activity have been obtained from arterial and tidal volume time-domain traces, respectively. FFT of \(\Delta P_{\text{tm}}\) was performed with Origin software (version 5.0; Microlab Software, Northampton, MA) by segmenting the most stable recordings obtained into blocks of 1,024 samples \((10.24 \text{ s of recording})\) and performing a baseline correction for each block to minimize artifactual direct current components.

Stereomicroscopic images were enhanced using Simple PCI software (Compix, Cranberry Township, PA).

RESULTS

Spontaneous ventilation. During the whole experiment, the anesthetized rats spontaneously breathed at a tidal volume of 4 ± 0.34 ml and a respiratory rate of 66 ± 2.9 cycles/min. Heart rate and mean arterial and venous pressures averaged 284.7 ± 9.5 cycles/min, 90.6 ± 4.2 mmHg, and 8.8 ± 1.3 mmHg, respectively. During the end-expiratory phase, at a chest wall volume corresponding to the functional residual capacity, esophageal pressure \((P_{\text{esoph}})\) was \(-1.5 \pm 0.7 \text{ mmHg}\) and decreased to \(-4.4 \pm 0.8 \text{ mmHg}\) at attainment of the end-inspiratory volume. The respiratory and the cardiovascular parameters remained essentially steady throughout the whole experimental period, slowly declining only after \(\sim 6\) h of deep general anesthesia.

In Fig. 2A, typical recordings of \(P_{\text{lymph}}\) (middle, continuous line) and \(P_{\text{int}}\) (middle, dotted line) obtained during spontaneous breathing at 93 cycles /min \((\sim 1.6 \text{ Hz})\) are presented. The
P<sub>lymph</sub> and P<sub>int</sub> traces are characterized by large pressure waves associated with the changes in tidal volume (top) during spontaneous respiratory swings. Small cardiogenic oscillations, associated with cardiac activity and amounting to −0.5 mmHg, also are detectable in the original P<sub>lymph</sub> trace. In the example in Fig. 2A, the end-expiratory (P<sub>lymph-E</sub>) and end-inspiratory (P<sub>lymph-I</sub>) intraluminal lymphatic pressure averaged −0.7 ± 0.2 and −3 ± 0.1 mmHg, respectively, whereas the end-expiratory (P<sub>int-E</sub>) and end-inspiratory (P<sub>int-I</sub>) intercostal interstitial pressures were 4 ± 0.5 and −2.8 ± 0.4 mmHg, respectively. Each of the above pressure values was obtained by averaging the corresponding pressure peaks in a time frame of up to 10 s.

When the data obtained from all readings were averaged, P<sub>lymph-E</sub> was −2.5 ± 1 mmHg (range: from −18.4 to 13.7 mmHg; n = 47) and was significantly lower (P < 0.01, one-way ANOVA) than the corresponding average P<sub>int-E</sub> amounting to 3 ± 0.7 mmHg (range: from −9.9 to 10.7 mmHg; n = 42). The corresponding average P<sub>lymph-I</sub> and P<sub>int-I</sub> values were −21.1 ± 1.3 mmHg (range: from −28 to −5.6 mmHg; n = 47) and −12.2 ± 1.3 mmHg (range: from −3 to 3.8 mmHg; n = 42), respectively. Therefore, the inspiratory maneuver caused both P<sub>lymph</sub> and P<sub>int</sub> to drop significantly (P < 0.001, one-way ANOVA) by 18.6 ± 1.8 and 15.3 ± 1.4 mmHg, respectively, compared with the corresponding end-expiratory values. Figure 2 clearly shows how ΔP<sub>tm</sub> (bottom; ΔP<sub>tm</sub> = P<sub>lymph</sub> − P<sub>int</sub>) is not constant from breath to breath but actually changes over time, shifting from −5.3 ± 0.5 mmHg at end expiration to 0.5 ± 0.3 mmHg at end inspiration.

In the attempt to verify whether two functionally different populations of lymphatic vessels could be distinguished on the basis of their absorptive (negative ΔP<sub>tm</sub>) or propulsive (positive ΔP<sub>tm</sub>) roles throughout the respiratory cycle, the end-inspiratory transmural pressure gradients (ΔP<sub>tm-I</sub>) were plotted as a function of the corresponding end-expiratory transmural pressure gradients (ΔP<sub>tm-E</sub>) in spontaneous ventilation. Negative ΔP<sub>tm</sub> values indicate the existence of a net hydraulic pressure gradient from the interstitium to the lymphatic lumen. Vice versa, positive ΔP<sub>tm</sub> values may support either retrograde fluid flux and/or lymph propulsion. According to the position of data points in the plot quadrants, four functional conditions may be recognized. In cases of data falling in quadrants 1 and 3, ΔP<sub>tm</sub> is negative or positive, respectively, during the whole respiratory cycle. In cases of data in quadrants 2 and 4, ΔP<sub>tm</sub> may instead be either negative during expiration and positive during inspiration (quadrant 2) or vice versa (quadrant 4). As a result of this wide functional variability, no significant linear regression (ΔP<sub>tm-I</sub> = −5.9 ± 0.3ΔP<sub>tm-E</sub>; r² = 0.05; n = 40) or correlation (correlation coefficient = 0.233; P = 0.147; n = 40) was found between the two variables.

An example of the P<sub>lymph</sub> and P<sub>int</sub> traces during mechanical ventilation at positive alveolar pressure in paralyzed respiratory muscles is proposed in Fig. 2B. At variance with what was observed during spontaneous ventilation (Fig. 2A), P<sub>lymph</sub> and P<sub>int</sub> both increased with increasing lung volume. In fact, on average, at end expiration with zero alveolar pressure, P<sub>lymph-E</sub> and corresponding P<sub>int-E</sub> averaged −3.1 ± 1.2 (n = 7) and 2.6 ± 2.3 mmHg 8 (n = 7), respectively, and were not significantly different from values previously observed during spontaneous breathing. During mechanical ventilation, P<sub>esoph</sub> averaged −1.6 ± 0.7 mmHg at end expiration and increased to 2.8 ± 0.8 mmHg at end inspiration.

An example of the P<sub>lymph</sub> and P<sub>int</sub> traces during mechanical ventilation at positive alveolar pressure in paralyzed respiratory muscles is proposed in Fig. 2B. At variance with what was observed during spontaneous ventilation (Fig. 2A), P<sub>lymph</sub> and P<sub>int</sub> both increased with increasing lung volume. In fact, on average, at end expiration with zero alveolar pressure, P<sub>lymph-E</sub> and corresponding P<sub>int-E</sub> averaged −3.1 ± 1.2 (n = 7) and 2.6 ± 2.3 mmHg 8 (n = 7), respectively, and were not significantly different compared with the values attained during spontaneous breathing. However, at the end-inspiratory peak attained with positive alveolar pressure, P<sub>lymph-I</sub> and P<sub>int-I</sub> significantly increased to 28.1 ± 7.8 (P < 0.001) and 28.2 ± 6.3 mmHg (P < 0.001), respectively, with an inspiratory tidal pressure change of 31.3 ± 8.5 and 25.6 ± 5.6 mmHg, respectively.

From the FFT analysis of the pressure signals (Fig. 4B), one may appreciate that although the major source of P<sub>lymph</sub> and P<sub>int</sub> oscillations during mechanical ventilation is the respiratory component, this is not true for the ΔP<sub>tm</sub>, whose value at the ventilatory frequency (1 Hz) does not substantially differ from that of other components, including the cardiogenic one (~3

![Figure 3: End-inspiratory transmural pressure gradients (ΔP<sub>tm-I</sub>) as a function of the corresponding end-expiratory transmural pressure gradients (ΔP<sub>tm-E</sub>) in spontaneous ventilation. Negative ΔP<sub>tm</sub> values indicate the existence of a net hydraulic pressure gradient from the interstitium to the lymphatic lumen. Vice versa, positive ΔP<sub>tm</sub> values may support either retrograde fluid flux and/or lymph propulsion. According to the position of data points in the plot quadrants, four functional conditions may be recognized.](http://ajpheart.physiology.org/content/289/7/H266)
DISCUSSION

The lymphatic vessels investigated in the present study belong to the pleural lymphatic network, which provides fluid and solute drainage from the pleural cavity, the subpleural interstitial space, and the intercostal muscles. These vessels are subject to cyclic displacements, related to the change in chest shape and volume during the respiratory activity. In such an experimental condition, the reliability of the methodology and the approach used for pressure measurements becomes crucial.

Methodological evaluation. The micropuncture technique used in the present study offers the great advantage of allowing measurements of hydraulic pressure in tissues and in microvessels that would not be otherwise accessible. However, pressure measurement might be affected by artifacts caused by possible distortion of the flexible pipette tip and change in recording site within the moving tissue. To avoid tissue distortion during insertion, the pipette was driven into the tissue and/or the lymphatic vessel at an angle of \( \sim 30° \) with respect to the tissue/vessel surface. This approach had been proven to be the least invasive during pipette insertion in steady intercostal interstitium (7). The similarity between present \( P_{\text{lymph-E}} \) values and those obtained in paralyzed rabbits (14) and between present \( P_{\text{int-E}} \) values and those recorded in spontaneously breathing rats using saline-filled catheters (16) provides an indirect validation of this micropipette insertion approach in relatively still tissues.

Judging from the stereomicroscope images during recording, inspiratory chest expansion caused an outward and cranial displacement of the tissues of the order of 200–300 \( \mu \)m. In some instances, the deformation of the pipette tip against the vessel wall or the tissue fibers caused saturation of the pressure signal, leading to rejection of the reading. However, when the pipette was inserted along the major axis of the lymph vessel, or parallel to it in the interstitium, distortion was minimized and a stable and reproducible recording was obtained over several minutes. This insertion technique also allowed us to clearly distinguish whether the tip was in the vessel or in the adjacent interstitium. In any case, the completely opposite response observed in \( P_{\text{lymph}} \) and \( P_{\text{int}} \) for similar tidal volume changes attained with active inspiration or passive lung inflation (Fig. 2, A and B) suggests that possible artifacts caused by the recording device might only marginally affect the actual values.

\( P_{\text{lymph}}, P_{\text{int}}, \) and \( \Delta P_{\text{tm}} \) during the respiratory cycle in spontaneous and mechanical ventilation. As observed in previous studies carried out using the same technique on paralyzed rabbits and rats, both the diaphragmatic (18) and the intercostal (14) lymphatic vessels of sizes ranging from 50 to 300 \( \mu \)m may be functionally regarded as initial lymphatics (22). In fact, the pressure oscillations caused by spontaneous contraction of the lymphatic smooth muscle cells, commonly observed in organs such as the mammalian mesentery or the bat wing (1, 22), were only very rarely encountered in the present and in our previous

Fig. 4. A: fast Fourier transforms (FFT) of tidal volume (\( V_t \)), \( P_{\text{lymph}}, P_{\text{int}}, \) and \( \Delta P_{\text{tm}} \) traces shown in Fig. 2A during spontaneous breathing. Note that the major frequency component of \( \Delta P_{\text{tm}} \) is represented by the respiratory swings at 1.6 Hz, with a much smaller component at \( \sim 3.2 \) Hz. The peak observed at \( \sim 0.3 \) Hz is artifactual and due to the slow drift observed in the \( P_{\text{int}} \) recording (see Fig. 2A). B: FFT of \( V_t, P_{\text{lymph}}, P_{\text{int}}, \) and \( \Delta P_{\text{tm}} \) traces shown in Fig. 2B during mechanical ventilation. Note that although the respiratory swings account for the most important frequency component of the \( P_{\text{lymph}} \) and \( P_{\text{int}} \) traces, the same is not true for \( \Delta P_{\text{tm}} \).

Hz). In addition, at variance with what was observed during spontaneous breathing (Fig. 4A), when the inspiratory \( \Delta P_{\text{tm}} \) peak was much higher than the corresponding \( P_{\text{lymph}} \) and \( P_{\text{int}} \) peaks, with mechanical ventilation the \( \Delta P_{\text{tm}} \) component was significantly reduced during inspiration.

On average, the net transmural hydraulic pressure gradient was \( -5.7 \pm 2.6 \) mmHg at end expiration (\( \Delta P_{\text{tm-E}} \)) and \( -0.1 \pm 5.9 \) mmHg at end inspiration (\( \Delta P_{\text{tm-I}} \)). Over the entire respiratory cycle, \( \Delta P_{\text{tm}} \) was \( -1.1 \pm 1.7 \) mmHg, not significantly different from zero and significantly lower (\( P = 0.005 \), one-way ANOVA) than the value observed in spontaneously breathing animals (\( \Delta P_{\text{tm}} = -6.8 \pm 1.2 \) mmHg).

Cardiogenic oscillations. The amplitude of the cardiogenic oscillations was very variable and determined, on average, a tidal increase of \( P_{\text{lymph-E}} \) and \( P_{\text{int-E}} \) of 5.5 \( \pm 2 \) (\( n = 21 \)) and 5.1 \( \pm 0.7 \) mmHg (\( n = 21 \)), respectively. Such cardiogenic \( P_{\text{lymph}} \) and \( P_{\text{int}} \) swings generated a \( \Delta P_{\text{tm}} \) amounting to \( -3.7 \pm 1.61 \) mmHg, a value not significantly different from that observed across the wall of lymphatic vessels running on the pleural surface of the muscular part of the paralyzed rat diaphragm (14, 18). When performing a FFT analysis of a time frame of typical \( P_{\text{lymph}}, P_{\text{int}}, \) and \( \Delta P_{\text{tm}} \) traces, it is possible to distinguish the prevalent component of pressure oscillation. For instance, Fig. 4A presents such an analysis performed on traces in Fig. 2A; one may appreciate that the respiratory component, at \( \sim 1.5 \) Hz, is the most relevant source of \( P_{\text{lymph}} \) and \( P_{\text{int}} \) pressure oscillation and, more significantly, of \( \Delta P_{\text{tm}} \), whereas the cardiogenic component (at \( \sim 3.2 \) Hz in this example) is distinguishable only in the \( P_{\text{lymph}} \) recording. Note that the significant peak observed in Fig. 4A at \( \sim 0.3 \) Hz is artifactual and is due to the slow drift observed in the \( P_{\text{int}} \) trace and thus calculated \( \Delta P_{\text{tm}} \) values (see Fig. 2A). Cardiogenic oscillations amounting to \( -2 \) mmHg also are visible in the \( P_{\text{int}} \) and \( \Delta P_{\text{tm}} \) traces in Fig. 2B, corresponding to the oscillation component at \( \sim 3 \) Hz in Fig. 4B.
studies (14, 18). Hence, from this standpoint, pleural lymphatics seem to functionally behave like the lymphatics network supplying other tissues that undergo high tissue stresses, such as the skeletal muscle (23, 24) and the myocardium (5). $P_{\text{int-E}}$ depends on the local stress exerted between the solid macromolecular elements in the tissue as well as on the water content of the tissue (1) as indicated by the progressive increase of $P_{\text{int}}$ with overhydration (9). Because intercostal microvasculature provides net fluid filtration into the tissue (15), the absorptive gradient required to maintain the interstitial fluid homeostasis, as well as the negative end-expiration $P_{\text{int}}$ value, is therefore set by the lymphatic drainage, which indeed provides absorptive $\Delta P_{\text{lm}}$ (Fig. 3, quadrants 1 and 4) in most vessels at end expiration.

Upon inspiration, variable mechanical stresses arise in the intercostal tissues, resulting from the simultaneous contribution of multiple mechanical factors: 1) the inspiratory contraction of the external intercostal muscles that run ventrally in the craniocaudal direction and likely exert uneven tangential stresses on the differently oriented parasternal or paracostal subpleural interstitial fibers; the contribution of the external intercostal muscles to lung inflation varies markedly in the rostrocaudal direction, with the inspiratory rib displacement more relevant in the rostral interspaces compared with the caudal ones (2, 3); and 2) the inspiratory decrease in pleural liquid pressure ($P_{\text{iq}}$), which exerts a further inward pull, acting perpendicularly to the pleural surface. Such a local mechanical complexity results in an increased local tensile stress, as suggested by the decrease in $P_{\text{lymph}}$ and $P_{\text{int}}$ observed during spontaneous inspiration. The similarity between $P_{\text{int}}$ and $P_{\text{lymph}}$ inspiratory swings suggests that the stress arising in the tissue during inspiration was entirely transmitted to the lymphatic vessel lumen through the solid matrix components. Indeed, the initial lymphatics are delimited by a highly compliant endothelial wall (22), which is not expected to modify the stress exerted by the tissue fibers to the lymphatic lumen via the anchoring filaments. The variable delay observed between $P_{\text{int}}$ and $P_{\text{lymph}}$ changes at the spontaneous inspiratory onset (Fig. 2A) is likely related to the viscoelastic properties of the matrix fibers though which mechanical transmission of tissue stress to the outer lymphatic wall takes place. Such a delay, usually not present in mechanical ventilation in paralyzed rats (Fig. 2B), may reflect both changes in tissue compliance during intercostal muscle contraction or relaxation and/or alternate muscle recruitment.

During spontaneous breathing, tissue stress magnitude seems to be more effective than respiratory frequency in determining $\Delta P_{\text{lm}}$ changes. Indeed, although $\Delta P_{\text{lm}}$ developing during cardiogenic oscillations may support lymph function in the paralyzed diaphragm (18), the amplitude of $\Delta P_{\text{lm}}$ induced by respiratory activity is much prevailing (Fig. 4).

From a fluid dynamics standpoint, a negative $\Delta P_{\text{lm}}$ value across the lymphatic wall potentially drives an interstitial to lymphatic fluid flow, thus promoting lymph formation. It is worth noting that the initial lymphatic vessel may be compared, from the mechanical standpoint, to a nonelastic collapsible vessel (4); therefore, the tensile stress exerted by the tissue fibers on the outer surface prevents vessel collapse despite the occurrence of a negative transmural pressure. In the majority (~70%) of the recordings (Fig. 3, quadrants 1 and 2) $\Delta P_{\text{lm}}$ was in favor of lymph formation during the end-expiratory phase.

Provided that lymph pressure waves caused by the intrinsic spontaneous contraction of the smooth muscles cells were not observed in the present study, the setting and maintenance of the end-expiratory $\Delta P_{\text{lm}}$ likely depend on cardiogenic oscillations, similar to those observed in the lymphatic diaphragmatic network of paralyzed rats (18). In intercostal tissues, the cardiogenic swings depend on 1) displacement of lung parenchyma and pleural liquid during cardiac motion and 2) mechanical transmission of arterial pressure waves along the elastic walls of the internal mammalian and intercostal arteries. Whereas ~60% of the lymphatic vessels contributed to lymph formation during the entire respiratory cycle (Fig. 3, quadrant 1), in ~40% of the vessels a positive $\Delta P_{\text{lm}}$ developed, at least transiently, suggesting the occurrence of either forward fluid propulsion and/or backflow toward the interstitial space. The actual direction of the fluid flux between the interstitium and the lymphatic lumen cannot be detected with the techniques used in the present study, and thus we cannot exclude the existence of lymphatic to interstitial fluid backflow. However, unidirectional flaplike valves located in the lymphatic vessel wall (primary valves) have been recently described in the lymphatics supplying the skeletal muscles (6, 24) and the diaphragm (17). Given that these valves seem to hinder fluid backflow, a positive $\Delta P_{\text{lm}}$ might reasonably be interpreted in terms of lymph propulsion between adjacent lymphangions rather than as an index of retrograde fluid flux.

In paralyzed rats mechanically ventilated at zero end-expiratory alveolar pressure, $P_{\text{int-E}}$, $P_{\text{lymph-E}}$, and $\Delta P_{\text{lm-E}}$ did not significantly differ compared with their values during spontaneous breathing, suggesting that end-expiratory muscle tone did not significantly affect, per se, the lymphatic function, which was guaranteed by the unaffected cardiogenic oscillations. However, at variance with spontaneous breathing, $P_{\text{int-I}}$ and $P_{\text{lymph-I}}$ both increased with passive lung inflation as a result of compressive tissue stress due to increased alveolar pressure. In this condition, the average $\Delta P_{\text{lm-I}}$ was essentially nullified. This finding indicates that tissue stress induced by the intercostal muscle contraction and not the change in chest wall volume is required to support lymphatic function, which in this condition may only rely on the unaffected but less efficient cardiogenic oscillations (Fig. 4B).

**Pleural fluid drainage.** In spontaneously breathing rats at approximately heart level, costal $P_{\text{iq}}$ averages approximately ~2 mmHg at end expiration and approximately ~3 mmHg at end inspiration, respectively (11, 16). Assuming that the present $P_{\text{lymph}}$ values reflect $P_{\text{lymph}}$ within the stomata (19) facing the pleural space, a net pressure gradient varying between approximately ~0.5 mmHg at end expiration and approximately ~18 mmHg at end inspiration, respectively, would develop between the pleural fluid and the stomata lumen. Therefore, during spontaneous breathing, lymph formation might occur through the whole respiratory cycle, and in particular upon inspiration. Pleural fluid drainage would instead be hindered during mechanical ventilation when $P_{\text{lymph}}$ becomes positive.

In summary, the analysis of $\Delta P_{\text{lm}}$ development during either the respiratory or cardiac cycle shows that the active contraction of inspiratory muscles and not the chest wall volume change is required to enhance lymph formation and progression in the thoracic lymphatics. The three-dimensional architecture of matrix macromolecules, their arrangement in the
intercostal spaces, and their mechanical properties seem to play
a fundamental role in determining and modulating lymph
formation and propulsion in these initial lymphatics under
spontaneous breathing. Given the importance of the lymphatic
system in controlling fluid homeostasis in the thoracic tissues
and in particular in the pleural cavity, mechanical ventilation
per se is therefore expected to determine an increase of thoracic
tissues hydration and pleural effusion. A similar situation may
well occur in the lung parenchyma, motivating the latent
subedematous condition often observed in lungs of patients
exposed to positive pressure ventilatory regimes.

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