Composition, biophysical properties, and morphometry of plasma membranes in pulmonary interstitial edema

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Received 20 November 2001; accepted in final form 27 January 2002

Am J Physiol Lung Cell Mol Physiol 282: L1382–L1390, 2002. First published February 1, 2002; 10.1152/ajplung.00447.2001.—We evaluated the changes in plasma membrane composition, biophysical properties, and morphology of pulmonary endothelial cells in anesthetized rabbits receiving 0.5 ml·kg−1·min−1 saline infusion for 180 min, causing mild interstitial edema. Plasma membrane fractions were obtained from lung homogenates with gradient centrifugation, allowing a sixfold enrichment in caveolin-1. In edematous lungs, cholesterol content and phospholipidic phosphorus increased by 15 and 40%, respectively. These data correlated with morphometric analysis of lungs fixed in situ by vascular perfusion with 2.5% glutaraldehyde, suggesting a relative increase in surface of luminal to interstitial front of the capillary endothelial cells, due to a convoluted luminal profile. In edematous lungs, the fraction of double-bound fatty acids increased in membrane lipids; moreover, the phosphatidylycholine/phosphatidylethanolamine and the cholesterol/phospholipid ratios decreased. These changes were consistent with the increase in fluorescence anisotropy of plasma membrane, indicating an increase in its fluidity. Data suggest that mechanical stimuli elicited by a modest (~4%) increase in extravascular water cause marked changes in plasma membranes that may be of relevance in signal transduction and endothelial cell activation.

MATERIALS AND METHODS

Chemicals. The reagents used (analytical grade) and high-performance thin-layer chromatography (HPTLC) plates (Kieselgel 60) were purchased from Merck (Darmstadt, Germany). 3-[Cyclohexylamino]-1-propanesulfonic acid (CAPS), Percoll, calmodulin, NADPH, EDTA, EGTA, phenylmethylsulfonyl fluoride (PMSF), terahydrobiopterin (BH4), and L-arginine were from Sigma Chemical (St. Louis, MO). L-[3H]arginine was obtained from NEN (Boston, MA).

Lung tissue sample preparation for the biochemical analysis of plasma membranes. Lung tissue samples were obtained from 10 adult New Zealand White rabbits (2.53 ± 0.3 kg body wt, mean ± SD) anesthetized with a cocktail of 2.5 ml/kg of 50% (wt/vol) urethane and 40 mg/kg body wt of ketamine injected into an ear vein. Subsequent doses of anesthetic were administered during the experiment, depending on the arousal of ocular reflexes. Animals were tracheotomized to allow spontaneous breathing. Samples were obtained from control animals (killed shortly after anesthesia and tracheotomy; n = 5) and from animals that received slow saline infusion through the right superior jugular vein (0.5 ml·kg−1·min−1) for 3 h to induce interstitial edema (n = 5). The chest was opened through a midsternal incision (6). Next we opened the pericardium and cannulated the pulmonary artery by advancing a catheter via the right ventricle. The catheter was secured to the ventricular wall, and another ligature was placed around the catheter at the root of the air-blood barrier (6, 20). These studies also showed that, despite a remarkable resistance of the pulmonary tissue to edema formation, the increase in interstitial fluid volume occurs as a consequence of fragmentation of proteoglycans, important macromolecules of the extracellular matrix (24, 25, 31, 32) controlling microvascular permeability and tissue mechanical resistance. We wished to further investigate the condition of mild interstitial edema because it represents a critical equilibrium between matrix degradation and deposition provided by cellular activation. We focused on cellular involvement in mild interstitial edema, particularly on the composition and fluidity of plasma membranes that are known to be sensitive to alterations in chemical and physical stimuli of the environment surrounding the cells (34, 36).

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THE ALVEOLAR AND CAPILLARY WALLS delimit the pulmonary interstitium, a thin compartment made of a fiber system, serving as a scaffold, and other macromolecules forming the capillary and the alveolar basement membranes. Extravascular water is distributed within the interstitial compartment of the air-blood barrier and is physiologically kept at a minimum volume to optimize gas diffusion. Recent work, based on an experimental model causing a mild form of pulmonary interstitial edema, allowed us to describe the mechanisms contributing to a tight control of extravascular lung volume in

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pulmonary artery. An overdose of anesthesia was given to the animal just before the perfusion. After the left atrium was sectioned, we perfused the lungs for 3–5 min at room temperature with mammalian Ringer solution (without calcium) containing nitroprusside (20 mg/ml). After this time, the lungs were flushed with 50 ml of 0.25 M sucrose and 20 mM tricine, pH 7.4, with protease inhibitors (solution A) (27). The lungs were then excised from the chest and immersed in ice-cold solution A with protease inhibitors. The lung tissue was then finely minced with a scalpel on a cold glass plate embedded in packed ice. The minute lung pieces were finally placed in a tube with 30 ml of solution A and homogenized.

**Purification of plasma membranes.** Lung homogenates obtained from all control (HC; 5 animals) and edematous lungs (HE; 5 animals) were subjected to Percoll gradient centrifugation to isolate a plasma membrane fraction (27). Briefly, the lung tissue was homogenized in buffer (0.25 M sucrose, 1 mM EDTA, 20 mM tricine, pH 7.8), and the lung homogenate was filtered sequentially through 53- and 30-μm filters. Aliquots of filtered tissue homogenates (HC and HE) were then taken for successive analysis, the remaining part of homogenate was subjected to centrifugation (1,000 g for 10 min) at 4°C, and the supernatant was saved. The resulting pellet was resuspended in 3 ml of buffer and subjected again to homogenization and centrifugation as above. The two supernatants were combined and overlaid over 25 ml of 30% Percoll in buffer after centrifugation using an SW28 rotor at 84,000 g for 45 min at 4°C, we collected a single membranous band readily visible about 5 cm from the bottom of the tube. To reduce the volumes and concentration the membranes, they were pelleted by diluting the suspension threefold with PBS before centrifugation at 100,000 g for 45 min at 4°C. The membrane fractions obtained from HC and HE were called PMC and PME, respectively, and aliquots were taken for different analysis.

The total protein content was assessed by bacitracin method (Sigma). The analysis of caveolin-1 was performed using the Bio-Rad Miniprotein II system, using 15% gel and monoclonal antibody (C2297, Transduction Laboratories), dilution 1:1,000.

**Phosphorus analysis and fluorescence spectroscopy.** Aliquots of PMC and PME from all animals were used for phospholipid phosphorus determination by the Bartlett (2) procedure. Data were expressed as micromoles per milligrams of protein.

The membrane fluidity of different samples was assessed by fluorescence anisotropy measurements of the fluorescent probe 1,6-diphenyl-1,2,5-hexatriene (DPH) as described (28) with minor modification. A suspension of PMC and PME containing ~200 nmol of phosphorus per 1.5 ml of PBS was used. The fluorescent probe molecule DPH was added to membrane suspension at a final concentration of 10−3 M. Light scattering was corrected by using a blank containing the sample but not DPH. Membranes with and without fluorescent probe were incubated in the dark under stirring for 45 min at 37°C and were used for fluorescence polarization studies immediately after preparation. A polarization spectrofluorimeter (Cary Eclipse, Varian) with fixed excitation and emission polarization filters was used to measure fluorescence intensity parallel (I_{pa}) and perpendicular (I_{per}) to the polarization plane of the exciting light (14). Excitation and emission wavelengths were 360 and 430 nm, respectively. Fluorescence anisotropy was calculated as r (I_{pa} - I_{per}/I_{pa} + I_{per}). The sample was continuously stirred with a microtirrer, and the temperature (37°C) was monitored by a thermostat in the cuvette.

**Measurement of nitric oxide synthase activity.** Nitric oxide synthase (NOS) activity was measured in HC and PMC (3 animals) and in HE and PME (3 animals) by monitoring the formation of L-[3H]citrulline from L-[3H]arginine (30, 42). Homogenates (200 μg of protein) and total plasma membranes (50 μg of protein) were incubated in 150 μl of 50 mM Tris buffer, pH 7.4, containing 0.1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 1 mg/ml leupeptin, 2.5 mM CaCl2, 1 mM NADPH, 100 nM calmodulin, 10 μM BH4, and 5 μM combined L-arginine and purified L-[3H]arginine (0.5 μM; sp act 54 Ci/mmol) at 37°C for 60 min. The reaction was terminated by heating samples at 90°C for 10 min. After being cooled, trichloracetic acid was added to a final concentration of 6% (wt/vol), and the precipitate was removed by centrifugation (17,000 g, 30 min). One microliter (carrying 6,000 pmol) of the supernatant was spotted on HPTLC silica gel plates. The HPTLC plates were developed in the solvent system chloroform:methanol:concentrated ammonia:water 1:4:2:4 (each by vol). Spots for L-[3H]arginine and L-[3H]citrulline were quantified with a β-Imager 2000 Instrument (Biospace, Paris, France) using the specific β-Vision software provided by Biospace.

The quantity of L-[3H]arginine converted to L-[3H]citrulline was calculated from the specific activity of L-[3H]arginine added to the assay mixture after subtraction of the blank values. The enzyme activity was expressed as picomoles of L-[3H]citrulline·h−1·mg−1 of protein.

**Lipids and fatty acid analysis.** Aliquots of HC and PMC (5 animals) and HE and PME (5 animals) were submitted to lipid extraction (40). An organic phase (containing all lipids with the exception of gangliosides) and an aqueous phase (containing gangliosides) were obtained. The lipids were separated on HPTLC plates. The phospholipids from PME and PME were chromatographed in solution B (chloroform:methanol:acetic acid:water, 60:45:4:2, each by vol). The gangliosides from PMC and PME were chromatographed in solution C (chloroform:methanol:0.2% CaCl2, 50:42:11, each by vol). The cholesterol, from both homogenates and plasma membranes, was chromatographed in solution D (hexane:diethyl ether:acetic acid, 20:35:1, each by vol). In the case of neutral glycosphingolipids (GLS), the lipids extracted were submitted to alkaline methanolysis (1 h at 37°C in 0.6 N NaOH in methanol) to remove contaminating phospholipids. After extensive dialysis, the GLS were chromatographed in solution E (chloroform:methanol:water, 110:40:6, each by vol). For the analysis of the plasmalogens, the phospholipids were chromatographed in solution B. The plates were then exposed to HCl vapors for 10 min and subsequently chromatographed in solution F for second dimension (chloroform:methanol:acetic acid:water, 50:15:15:10:5, each by vol). Phospholipids and cholesterol were visualized with anisaldehyde, gangliosides with resorcinol (37), and neutral GLCs with orcinol (39). The plates were scanned with Bio-Rad system and spot identification, and quantification was accomplished by comparison with authentic standard lipids.

Aliquots of different total lipids extracted, corresponding to 100–150 nmol of phosphorus, were submitted to fatty acid analysis (11). Briefly, lipid fatty acids were converted to fatty acid methyl esters in 0.5 N HCl in methanol for 20 h at 80°C. Fatty acid methyl esters were extracted into hexane and analyzed by gas-liquid chromatography (HP-INNOWax capillary column, PEG cross-linked 30 m×0.32 mm×0.5 μm; temperature program: 150°C for 1 min, 8°C/min from 150 to 200°C, 2°C/min from 200 to 250°C for 20 min).

**Lung tissue sample preparation for electron microscopy and morphometry.** In nine additional rabbits (2.57 ± 0.5 kg body wt, mean ± SD), we performed an ultrastructural mor-
phometric study to evaluate the changes in cell profile morphology. Lung tissue samples were obtained from control animals (killed shortly after anesthesia; n = 2), sham animals (kept under anesthesia for 3 h; n = 3), and animals in which interstitial edema was induced by saline infusion (0.5 ml·kg·min⁻¹ for 3 h; n = 4). The animals were anesthetized and tracheotomized as described above. The pulmonary artery was cannulated using the surgical approach previously described (6). The surgical preparation allows the pleural sacs to be left intact, therefore maintaining the physiological expansion of the lungs. Perfusion was carried out from two reservoirs arranged in parallel, connected to the pulmonary artery, and placed at a height of 15 cm relative to the right atrium that was snipped to allow the drainage of the perfusate. The reservoirs contained saline-dextran (11.06 g NaCl/l, 350 mosM, pH 7.4) for 2 h at 4°C, dehydrated in an ethanol scale, and animals in control and edematous lungs averaging 0.92 ± 0.08, respectively. The water content of the lung tissue was estimated from the wet to dry weight ratio of lung specimens that averaged 5.3 ± 0.3 and 5.5 ± 0.2 in control and edematous lungs, respectively (4% not significant increase). The dry weight of the lung tissue was estimated by dividing the wet weight by the wet-to-dry weight ratio; it was not significantly different in control and edematous lungs averaging 0.92 ± 0.09 and 0.89 ± 0.08, respectively.

The hematocrit value decreased significantly from 43 ± 2 in control to 36 ± 2 in edematous lungs, reflecting plasma dilution.

**Morphometric analysis.** Cell population of the alveolar septa includes three main cell types: endothelial, epithelial, and interstitial cells (10). Morphological and morphometric analysis was focused on the endothelial and epithelial (alveolar type I) cells in lung parenchyma because they represent the boundary of the alveolar tissue with blood and air, respectively, and therefore display the highest Sv among the cell populations in the septa (7, 13).

Figure 1 shows the ultrastructural appearance of lung parenchyma in control (A) and edematous animals (B). It appears clearly that in control lungs, the endothelium and epithelium are made of thin and smooth cytoplasmic sheets separated by a thin interstitial layer, the three layers together forming the air-blood barrier. In interstitial edema, endothelial cells present an irregular profile with plasma membrane invaginations, mainly on their luminal front. Epithelial cells also show some irregularities on the alveolar front. Note also the enlargement of the basement membranes in edema. The surface irregularities and invaginations of cell profiles on luminal and ablue
minal sides of endothelial and epithelial cells could be well identified with a magnification of \( \times 15,000 \).

At the relatively high magnification required for morphometric analysis, a strictly random sampling of the lung parenchyma was not practical because the number of micrographs mostly occupied by air with absence or a very small amount of tissue would have been very large. Therefore, we selected, at lower magnification, the areas of the lung parenchyma that contained capillaries and then increased the magnification to the desired level at which the morphological features of the cell surface profile could be well identified. Although the \( V_v \) and \( S_v \) we calculated are not representative for the whole lung, they are to be considered valid for the sample studied.

No differences were found when comparing both \( S_v \) and \( V_v \) estimates from control and sham animals; therefore, the corresponding data were averaged to form the control group. Point counting volumetry was used to determine the degree of interstitial fluid accumulation in edema. The relative \( V_v \) of the cell and of the extracellular interstitial compartments were \( 0.84 \pm 0.06 \) and \( 0.16 \pm 0.06 \) in control lungs, respectively; the latter value significantly increased in interstitial edema to \( 0.25 \pm 0.08 \) (\( P < 0.05 \)).

Interstitial edema led to an increase in the number of intersections per micrograph for the luminal endothelial front (Table 1). Furthermore, Table 1 also shows a significant increase in the ratio of the luminal endothelial intersections to the total intersections, indicating that luminal membrane is relatively increased compared with the interstitial membrane. No changes were observed when comparing control with edema for the alveolar and interstitial front of the epithelial cells. Figure 2 reports the \( S_v \) values with respect to the alveolar tissue volume. In this case, the changes in \( S_v \) when comparing control with edema are influenced both by the changes in surface area and in volume of the alveolar tissue. The estimates of \( S_v \) were significantly decreased in edema relative to control, except for the luminal endothelial front.

Comparison of homogenate to plasma membrane fraction. We determined the content of caveolin-1 that represents a good marker of plasma membrane. We found that it increased about sixfold when comparing homogenate to plasma membrane fraction, thus proving that the purification yielded a satisfactory enrichment.

Measurement of NOS activity. The activity of NOS (Table 2) was increased sevenfold when comparing HC to PMC, in line with the expected enrichment of plasma membrane fraction. It significantly increased about twofold in PME with respect to PMC.

Phosphorus analysis and fluorescence spectroscopy. The content of phospholipidic phosphorus in plasma membranes was significantly increased in edematous lungs (Table 2). Using DPH, a fluorescent probe of the membrane fluidity, a significant decrease of the anisotropy parameter \( r \) was detected in PME (Table 2), indicating an increase in fluidity.

Table 1. Morphometric counts per lung tissue sample micrograph

<table>
<thead>
<tr>
<th></th>
<th>( L_v ) Lum</th>
<th>( L_v ) Int</th>
<th>( L_v ) Lum/( L_v ) Tot</th>
<th>( L_v ) Int/( L_v ) Tot</th>
<th>( L_v ) Alv</th>
<th>( L_v ) Int</th>
<th>( L_v ) Alv/( L_v ) Tot</th>
<th>( L_v ) Int/( L_v ) Alv</th>
<th>( L_v ) Int/( L_v ) Alv</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>29.5 ( \pm ) 10.7</td>
<td>25.1 ( \pm ) 8.3</td>
<td>0.540 ( \pm ) 0.039</td>
<td>0.459 ( \pm ) 0.039</td>
<td>27.2 ( \pm ) 10.7</td>
<td>23.9 ( \pm ) 8</td>
<td>0.525 ( \pm ) 0.046</td>
<td>0.475 ( \pm ) 0.046</td>
<td>0.475 ( \pm ) 0.046</td>
</tr>
<tr>
<td>Edema</td>
<td>34.8 ( \pm ) 15.5*</td>
<td>24.6 ( \pm ) 12.6</td>
<td>0.587 ( \pm ) 0.095†</td>
<td>0.413 ( \pm ) 0.095†</td>
<td>28.1 ( \pm ) 15.8</td>
<td>24.1 ( \pm ) 11.8</td>
<td>0.529 ( \pm ) 0.069</td>
<td>0.471 ( \pm ) 0.069</td>
<td>0.471 ( \pm ) 0.069</td>
</tr>
</tbody>
</table>

Data are means \( \pm \) SD. *\( P = 0.08 \); †\( P < 0.05 \) vs. control. L, number of cell profile intersections with the grid horizontal test lines per micrograph recorded in parenchyma; en, endothelium; ep, epithelium; Lum, luminal front; Int, interstitial front; Alv, alveolar front; en tot, lum + int; ep tot, alv + int.

Fig. 1. Micrographs of the air-blood barrier in control lungs (A) and in mild interstitial edema (B). Note the invagi-nations and irregularities of the endothelial cell profile on the luminal front as well as the thickening of the base-ment membranes in interstitial edema. Ep, epithelial cell; en, endothelial cell; cap, capillary. Scale bar = 1 \( \mu m \).
Lipid analysis. Aliquots of samples were submitted to lipid extraction, and the different lipids (cholesterol, ganglioside, neutral glycolipid, and phospholipid) were separated on HPTLC plates. The cholesterol concentration, normalized to total protein content (Fig. 3), doubled when comparing HC with PMC, in line with the enrichment procedure. A significant increase in cholesterol content was observed when comparing PMC with PME. In control lung plasma membranes, the analysis of gangliosides revealed that the more abundant ganglioside was GM3 (45%), followed by GD1a (17%), GM1 (15%), and GD3 (13%) [ganglioside nomenclature is according to Svennerholm (38)]. No changes were found in acid GLS plasma membrane composition when comparing control with edematous lungs.

Some differences were found in the pattern of neutral glycolipids obtained from plasma membranes (Fig. 4). The most abundant glycolipid, the lacto-N-neotetraosylceramide (tetra in Fig. 4), decreased in PME.

Table 2. Nitric oxide synthase activity, phospholipidic phosphorus content, and fluorescence anisotropy parameter

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Interstitial Edema</th>
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<tbody>
<tr>
<td><strong>Nitric Oxide Synthase Activity, pmol·mg protein⁻¹·h⁻¹</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>61 ± 3.0</td>
<td>63.5 ± 3.2</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>472 ± 8.9</td>
<td>945 ± 54*</td>
</tr>
<tr>
<td><strong>Content of phospholipid phosphorus, μmol/mg protein</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>1.09 ± 0.2</td>
<td>1.53 ± 0.3†</td>
</tr>
<tr>
<td><strong>Fluorescence anisotropy values of DPH, r</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>0.250 ± 0.009</td>
<td>0.236 ± 0.004#</td>
</tr>
</tbody>
</table>

Data are means ± SD. *P < 0.001, †P < 0.05 vs. control. DPH, 1,6-diphenyl-1,2,5-hexatriene; r, fluorescence anisotropy.
relative to PMC, whereas triosylceramide (tri in Fig. 4) increased significantly.

The phospholipid pattern is shown in Fig. 5A. When comparing plasma membrane preparation with control and edematous lungs, the phosphatidylethanolamine (PE) increased by ~20% while phosphatidylserine (PS) decreased by a similar amount. Phosphatidylglycerol content was similar in control and treated lungs, averaging ~3% (data not shown). When expressing phospholipids normalized to protein content (Fig. 5B), a significant increase in all phospholipid types was observed except for PS. The concentration of choline plasmalogen [included in phosphatidylcholine (PC)] was essentially unchanged when comparing control with edematous lungs. Furthermore, its percentage fraction of total PE remained unchanged (~8%). The concentration of ethanolamine plasmalogen (included in PE) increased by ~50% in interstitial edema, although its percentage fraction of total PE remained unchanged (~45%).

**Fatty acid analysis.** Table 3 reports the percentage composition of total lipid fatty acids from plasma membranes. In edematous lungs, an increase of linoleic (18:2) and arachidonic (20:4) acids was found, whereas the palmitic acid (16:0) decreased, leading to a significant decrease in double-bound index (DBI) from 0.659 ± 0.008 to 0.503 ± 0.01 (P < 0.01).

**DISCUSSION**

Slow-rate saline infusion leads to mild pulmonary interstitial edema due to an increase in microvascular filtration pressure gradient (due to decreased plasma colloid osmotic pressure) and in filtration area (due to greater lung perfusion). This experimental model has been extensively used in previous work because it offers the advantage to study the sequence of the early events accompanying fluid accumulation in the extravascular space. It was found that the air-blood barrier provides a strong safety factor against the development of severe edema because of its structural resistance. In fact, despite some interstitial matrix damage, it essentially assures low microvascular permeability and low tissue compliance (20). Pulmonary interstitial edema is characterized by a small increase in extravascular water (~4%) and by marked changes in the forces acting on cell surfaces at the interstitial level, due to matrix swelling, and possibly also on the luminal side of endothelial cells, due to variations in shear stress accompanying changes in vascular perfusion.

The present data indicate that mild pulmonary interstitial edema caused marked changes in lipid composition and fluidity of plasma membranes as well as in morphology of endothelial cell profiles. In accordance with our recent work (6), the morphometric analysis did not show differences between sham and control animals, suggesting that the observed changes should

![Fig. 5. Percentage (A) and content (B) of phospholipids in plasma membrane fraction obtained from control (PMC) and edematous (PME) lungs. Data are given as means ± SD. SPH, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidyl-}

**Table 3. Fatty acid composition of total lipids in plasma membrane fraction**

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Control</th>
<th>Interstitial Edema</th>
</tr>
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<tbody>
<tr>
<td>14:0</td>
<td>1.57 ± 0.37</td>
<td>1.54 ± 0.21</td>
</tr>
<tr>
<td>16:0</td>
<td>38.04 ± 0.58</td>
<td>32.37 ± 0.9*</td>
</tr>
<tr>
<td>16:1</td>
<td>2.80 ± 0.22</td>
<td>2.93 ± 0.58</td>
</tr>
<tr>
<td>18:0</td>
<td>13.90 ± 1.8</td>
<td>13.30 ± 1.7</td>
</tr>
<tr>
<td>18:1</td>
<td>18.58 ± 0.9</td>
<td>18.80 ± 1.8</td>
</tr>
<tr>
<td>18:2</td>
<td>13.20 ± 2.3</td>
<td>15.90 ± 1.6†</td>
</tr>
<tr>
<td>20:0</td>
<td>0.77 ± 0.25</td>
<td>0.78 ± 0.03</td>
</tr>
<tr>
<td>20:1</td>
<td>0.41 ± 0.01</td>
<td>0.46 ± 0.05</td>
</tr>
<tr>
<td>20:4</td>
<td>9.05 ± 0.2</td>
<td>11.13 ± 2.86*</td>
</tr>
<tr>
<td>22:0</td>
<td>0.74 ± 0.04</td>
<td>1.01 ± 0.27</td>
</tr>
<tr>
<td>22:1</td>
<td>0.47 ± 0.1</td>
<td>1.05 ± 0.66</td>
</tr>
<tr>
<td>24:0</td>
<td>1.57 ± 0.15</td>
<td>1.73 ± 0.15</td>
</tr>
<tr>
<td>24:1</td>
<td>0.94 ± 0.11</td>
<td>1.24 ± 0.29</td>
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**Double-Bound Index**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Interstitial edema</th>
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<tbody>
<tr>
<td></td>
<td>0.659 ± 0.008</td>
<td>0.503 ± 0.01†</td>
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</table>

Data are means ± SD. *P < 0.05; †P < 0.01 vs. control.
depend on the disturbance in interstitial fluid dynamics. Therefore, we were prompted to use only animals killed shortly after anesthesia as control animals for the biochemical analysis.

The purification methods used led to an enrichment of typical plasma membrane components, like caveolin-1, the principal integral protein present in flask-shaped plasmalemma invaginations 50–100 nm in diameter, called caveolae (35). Accordingly, the methods should be considered adequate to study plasma membrane composition. This is also confirmed by the increase of NOS activity and cholesterol content when comparing homogenate to plasma membrane fractions. Concentrations of different plasma membrane components were normalized to total protein content. This appears a valid reference, considering that the dry weight of the lung did not change in interstitial edema.

The increase in plasma membrane fluidity in edematous lungs can be correlated with the decrease of the PC:PE ratio from 1.04 to 0.9, with the cholesterol: phospholipid ratio from 0.22 to 0.185, and also with the decrease of the DBI value from 0.659 to 0.503. These data are in accordance with those obtained in cultured cells and tissues (1, 5, 16, 29). The increase of membrane fluidity can also be explained by a change in fatty acid lipid composition that causes an increase in free volume between the lipid chains, due to the presence of double bounds in fatty acids (18:2 and 20:4), leading to a bend in the lipid chain itself. Plasmalogens have been described to increase with oxidative stress and were also proposed as effective endogenous antioxidants (4). In fact, changes in mechanical stimuli (shear stress) acting on endothelial cells were shown to induce generation of reactive oxygen species (9). Because we found a change in ethanolamine plasmalogen content, we might deduce that mild pulmonary interstitial edema may imply oxidative stress.

The increase in plasma membrane cholesterol and phosphorus content, normalized to total protein, suggests an increase in membrane surface area. The observed changes could be reasonably ascribed to endothelial and epithelial (alveolar type I) cells because they provide most of the surface extension of the air-blood barrier.

An indication of the relative changes in plasma membrane surfaces in interstitial edema may be derived from data in Table 1. The increase in the number of intersections per sample micrograph for the luminal front of endothelial cells as well as the increase in the ratio of the luminal endothelial intersections to the total intersections (luminal + interstitial) indicate that capillary luminal membrane may be relatively increased. The decreased $S_v$ of endothelial and epithelial membranes in edema when referred to alveolar tissue is ascribed to an increase in alveolar tissue volume. This is consistent with a 4% increase in extravascular lung water and the observation that, using the same experimental model (6), a relevant portion (44%) of the extravascular fluid was shown to accumulate in the interstitial compartment of the alveolar septa, causing an ~30% increase in thickness of the air-blood barrier. The latter point is in line with the increase in $V_0$ of the extracellular matrix from 16 to 25% in the alveolar tissue reported in the present study.

More specifically, one can note that the decrease in $S_v$ is larger and significant for the interstitial compared with the luminal endothelial front, in line with a higher surface expansion of the luminal endothelial profile in edema.

The $S_v$ values reported in Fig. 2 are larger than the commonly reported values for the endothelial and epithelial cells that are in the range of 6,000–7,000 cm²/cm³ of alveolar tissue (10, 13). The discrepancy between the present and the reported values could well be accounted for by the difference in sampling procedure and in the final magnification used (7, 13). A high resolution is required to correctly identify surface details.

The increase in plasma membrane phospholipids is likely to reflect an increased synthesis during the experimental study. This would appear consistent with the demonstration that in central nervous system and liver, lipid synthesis is triggered within a relatively short time (33, 41).

The plasma membrane fraction from lung tissue might also include surfactant coming from alveolar surface and from lamellar bodies of alveolar type II cells. The increase in PC (Fig. 5B) that represents an important component of surfactant phospholipids and of caveolae suggests that interstitial edema may cause an increase in vesicle formation and/or in surfactant turnover.

We may attempt to relate biochemical and biophysical changes in plasma membranes to the changes in physical stimuli acting on endothelial cell surface. The increase in tissue forces at interstitial level is a consequence of interstitial fluid accumulation, particularly in the air-blood barrier as demonstrated by the increased $V_0$ of the extracellular matrix. Interstitial swelling causes an increase in interstitial fluid pressure from the physiological value of approximately $-10$ cmH₂O up to $-5$ cmH₂O (20). Because in mild interstitial edema, capillary pressure remains essentially unchanged at $-10$ cmH₂O (21, 23), the above condition should cause a substantial change in transmural pressure acting across the endothelial cells. In fact, in physiological conditions, the transmural pressure is relatively high, averaging 20 cmH₂O, the subatmospheric interstitial pressure pulling on the basal side of the cell. In edema, on the contrary, the transmural pressure gradient is reduced down to 5 cmH₂O, and the positive interstitial pressure pushes on the basal side of the cell. Furthermore, parenchymal forces exerted at focal points where cell-matrix attachments occur are expected to increase as a consequence of swelling of the extracellular matrix. Morphometry actually revealed only a slight, although not significant, change in $S_v$ of endothelial cells on the basal side, reflecting a still regular interstitial profile. This could be explained by the firm attachment of the cells to the basement membranes and by the strong structural resistance of the basement membranes in the air-blood barrier (6).
Furthermore, a change in shear stress could result from the vascular conditions caused by saline infusion. In a laminar flow regime, shear stress at the vessel wall (τ) is given by

\[ \tau = \frac{8 \cdot \text{vel} \cdot \eta_b}{D}, \]

where \( \text{vel} \) is the average flow velocity, \( \eta_b \) is blood viscosity, and \( D \) is the average vessel diameter. We may attempt an estimate of the change in \( \tau \), considering the dependence of \( \eta_b \) from plasma viscosity (\( \eta_p \)) and hematocrit (Ht), given by \( \eta_b = \eta_p \cdot (1 + 2.5 \text{ Ht}) \). \( \eta_b \) would decrease by ~9% for an ~16% decrease in Ht. Cardiac output increased by ~40%, relative to control (20), as previously measured in similar experiments. Assuming an increase in velocity in the microvascular segment similar to that found for the cardiac output, the total increase in \( \tau \) would not exceed 30%. However, temporal and spatial variations of shear stress ought to be expected due to the difference in vasomotor response of microvessels (22) and also in luminal profile displaying surface irregularities. In fact, the assumption of a laminar flow regime may not be valid with such a complex surface morphology. Shear stress itself was shown to alter surface topography of cultured endothelial cells, leading to reorganization of the cytoskeleton and adhesion sites (8).

Using a fluorescence probe, Haiddekker and coworkers (12) hypothesized that hydrodynamic shear destabilizes the plasma membrane, leading to an increase in fluidity. The membrane alterations could activate membrane-associated signaling proteins, for example heterotrimeric G proteins (15, 19) and endothelial NOS (18, 26). The product of this enzyme, nitric oxide, is a widespread mediator in physiology and functions in the circulatory system as an endogenous vasorelaxant (17). In our experiments, NOS activity doubled in plasma membranes from edematous lungs, suggesting a possible functional relationship to the change in membrane fluidity. Moreover, the increase in NOS activity, in addition to the altered fatty acid profile of plasma membranes [increase of arachidonic acid (20:4)], suggests the activation of transmembrane signaling pathways of the inflammatory process. This is in line with the previous observation demonstrating in mild interstitial edema the activation of metalloprotease-9 of neutrophil origin that cleaves matrix proteoglycans (31, 32).

In summary, although pulmonary interstitial edema implies a modest change in extravascular water, it likely entails mechanical stimuli influencing signal transduction either at cell surface or through the cytoskeleton leading to the release of cytokines and other mediators. Endothelial cells, representing the interface sensing both vascular and parenchymal signals, may be primarily involved to trigger transcription factors and protein expression that in turn influence either matrix deposition and tissue repair or, conversely, activate proteases leading to severe tissue damage.

The authors thank Prof. G. Bernocchi for useful discussion, Emanuela Clementi for help with preparation of ultrastructural sections, and Dr. Ombretta Bruschi for help with morphometric analysis. This research was supported by Cofinanziamento, Ministero dell’ Università e della Ricerca Scientifica e Tecnologica 1999 (to P. Palestini and G. Miserochi).

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