Albumin uptake and transcytosis in endothelial cells in vivo induced by albumin-binding protein

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Vogel, Stephen M., Richard D. Minshall, Milena Pili-povic, Chinnaswamy Tiruppathi, and Asrar B. Malik. Albumin uptake and transcytosis in endothelial cells in vivo induced by albumin-binding protein. Am J Physiol Lung Cell Mol Physiol 281: L1512–L1522, 2001.—The 60-kDa endothelial cell surface albumin-binding glycoprotein (gp60) is postulated to be a docking site for albumin that mediates the uptake of albumin and its transport in cultured microvessel endothelial cells. In the present study, we used an isolated Krebs-perfused rat lung preparation to address the in vivo role of gp60 in mediating albumin uptake and transport. Addition of primary anti-gp60 antibody to the perfusate followed by the secondary antibody to cross-link gp60 increased the vessel wall 125I-albumin permeability-surface area (PS) product 2.5-fold without affecting the capillary filtration coefficient ($K_{f,c}$, a measure of liquid permeability). In contrast, EDTA (5 mM), which induces interendothelial gap formation, produced parallel increases in both $K_{f,c}$ and 125I-albumin PS product. Increasing perfusate albumin concentration to >1 g/100 ml (EC50 1.2 g/100 ml) was sufficient to block 125I-albumin PS product, indicating that the perfusate albumin competed with tracer albumin for transendothelial albumin transport. Cross-linking of gp60 in lungs perfused with saturating concentration of albumin resulted in a greater increase in 125I-albumin PS product, indicating that gp60 function was capable of being modulated. These results show that activation of gp60 in pulmonary microvessels induces albumin uptake and its transport through a nonhydraulic pathway that fits with a model of albumin permeability via the transcellular pathway.

Serum albumin has critical functions in maintaining the transendothelial oncotic pressure gradient (and hence in regulating tissue fluid balance) and in transporting an array of bloodborne substances, among them lipids, hormones, peptides, nucleic acids, and drugs (9, 23–25). Albumin at concentrations as low as 0.05 g/100 ml maintains the endothelial barrier characteristics through interactions with extracellular matrix components (6, 14, 27). In addition, albumin can cross the vascular endothelial barrier, in part, by a diffusional paracellular pathway that can be augmented with the formation of interendothelial gaps (12, 17). Agents such as EDTA increase paracellular permeability through chelation of intercellular Ca$^{2+}$ and Mg$^{2+}$ (10, 16). Inflammatory mediators such as thrombin and histamine can also increase this pathway through activation of the endothelial actin-myosin cytoskeletal system (11, 18).

The question whether a transcellular pathway for albumin permeability is an important mechanism of transport in pulmonary microvessels remains uncertain. Tiruppathi et al. (35) and others (30) showed that albumin can bind specifically and reversibly to the endothelial cell surface glycoprotein gp60, a docking molecule for native albumin that initiates the transport of albumin in endothelial monolayers. gp60 is one of several endothelial albumin-binding proteins that may regulate albumin uptake and transport (1). Tiruppathi et al. (36) showed in endothelial cell monolayers that activation of gp60 by cross-linking with an anti-gp60 antibody stimulated transendothelial albumin transport without affecting the paracellular pathway as determined by endothelial monolayer hydraulic conductivity and transendothelial electrical resistance. They (36) also showed that gp60 activation induced the activation of Src kinases, suggesting that this is an early signaling event in endocytosis of albumin.

In the present study, we show that activation of gp60 (cross-linking of antigen with specific anti-gp60 antibody plus secondary antibody) stimulates the endothelial albumin uptake and transendothelial permeability of albumin in pulmonary microvessels. This stimulation of albumin transport was dissociated from the changes in liquid permeability, indicating that the gp60-mediated transport utilizes a nonhydraulic pathway in traversing the endothelial barrier. Moreover, gp60 function could be modulated such that its cross-linking increased transendothelial 125I-albumin permeability even in the presence of a saturating concentration of unlabeled albumin in the lung perfusate.
METHODS

Experimental Reagents

Antibodies. The detailed methodology for purification of gp60 from bovine lungs, immunization of rabbits with purified gp60, and extraction of polyclonal anti-gp60 antibodies from immunized serum is given by Tiruppathi et al. (35). IgGs were stored in 1-ml aliquots at −70 °C and thawed just before the experiments. Secondary antibodies (goat anti-rabbit IgG) were purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD).

Fluorescent probes. A polyclonal anti-gp60 antibody was labeled with indocyanine (Cy3)-bisfunctional reactive dye (Amersham Life Sciences, Pittsburgh, PA) according to the manufacturer's instructions. Alexa 488-labeled bovine serum albumin and LysoTracker, a lysosome marker, were obtained from Molecular Probes (Eugene, OR).

Iodinated albumin. Bovine serum albumin (fraction V, 99% pure and endotoxin free; Sigma-Aldrich, St. Louis, MO) was labeled with Na125I (NEN, Boston, MA) with the chloramine T procedure (5). Free 125I was separated from contaminant 125I in the tracer used contributed at a density of 1 NY). For monolayer culture (28), the cells were resuspended (RPMECs) were obtained from Vec Technologies (Rensselaer, NY). For testing the uptake and permeability of fluorescent probes, rat primary pulmonary vascular endothelial cells were cultured in RPMI medium (containing 10% fetal bovine serum, 2 mM L-glutamine, 40 μg/ml of gentamicin, 100 U/ml of penicillin, and 100 μg/ml of streptomycin) and incubated (37°C) under a humidified atmosphere of 5% CO2-95% air. Confluent monolayers formed on culture dishes or glass coverslips within 24–48 h. Monolayers used for experiments gave uniform, positive staining for factor VIII-related antigen.

Cell surface gp60 cross-linking in endothelial cells. Cultured RPMECs adhering to no. 1 glass coverslips were serum deprived for 2 h, washed two times with albumin (10 mg/ml)–Hanks' balanced salt solution (HBSS) at pH 7.4, and placed on ice for 1 h. For cross-linking gp60, the cell monolayers were cold incubated (4°C) for 15 min with anti-gp60 antibody (Ab; 10 μg/ml) plus, in some instances, a secondary Ab (goat anti-rabbit IgG, 10 μg/ml) to enhance cross-linking of antigen; unbound antibody was removed by additional cold rinses. On rewarming (37°C), gp60 was activated as previously described (35). Rewarming solutions contained various fluorescent probes (see Uptake of fluorescent probes). For control purposes, the monolayers were treated similarly except for the substitution of an isotype-matched control Ab (10 μg/ml) for the anti-gp60 Ab.

Uptake of fluorescent probes. For testing the uptake and internalization of certain fluorescent probes (alone or in combination), the rewarming solution (see Cell surface gp60 cross-linking in endothelial cells) contained the styryl pyridinium fluorescent dye RH 414 (5 μg/ml), Alexa 488-labeled bovine serum albumin (50 μg/ml), Cy3-labeled anti-gp60 antibody (3.5 μg/ml), and/or LysoTracker (50 nM). For fluorescent marking of endothelial cell nuclei, the monolayer cultures were rinsed, fixed with 4% paraformaldehyde in HBSS, and treated for 30 min in HBSS containing 5% goat serum, 0.1% Triton X-100, and 0.01% NaN3. The nuclear stain 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; 1 μg/ml) was added immediately after cell fixation. Unincorporated probe was removed by rinsing. Internalized fluorescent probes were viewed by confocal microscopy.

Fluorescence-activated cell sorter analysis. Confluent rat endothelial cell monolayers were washed with serum-free medium and incubated with the same medium for 2 h. After incubation, the cells were washed with PBS and detached by incubation with nonenzymatic cell dissociation medium (10–25 mg/ml) for 5 min at 37°C. Non-specific binding was blocked by incubating the cells for 60 min with 10% horse serum in ice-cold PBS. Cells (106/tube) were incubated with either gp60 antibody or preimmune serum (1:10 dilution, 60 min at 4°C), washed, and treated with FITC-conjugated goat anti-rabbit IgG for 30 min. After being washed, the cells were fixed with 1% paraformaldehyde and analyzed with an flow cytometer (Coulter EPICS Elite ESP; Coulter, Hialeah, FL). The mean logarithmic fluorescence intensity for each sample was determined and converted into linear relative fluorescence units (ΔFL) with the formula ΔFL = 10[E−0.0137], where E is the mean channel fluorescence intensity (19).

Lung Experiments

Krebs-perfused lung preparation. With prior approval from the University of Illinois at Chicago College of Medicine Animal Care Committee, Sprague-Dawley rats weighing 325–350 g were anesthetized with vaporized halothane (3% in 20% O2-80% N2), which was delivered (at 2 l/min) into a bell jar for induction and then into a specially designed nose cone. A cannula was inserted into a slit in the exposed trachea and connected to a Harvard small-animal ventilator, allowing positive-pressure ventilation of the lungs (tidal volume 3 ml; rate 40 breaths/min) and continued administration of the anesthetic gas. The right jugular vein was injected with 400 units of heparin for anticoagulation. A thoracotomy was performed, and within 15 s, the pulmonary artery was cannulated in situ for perfusion of the lungs with a modified Krebs-Henseleit solution (composition in mM: 118 NaCl, 4.7

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KCl, 1.0 CaCl₂, 1.0 MgCl₂, 5.0 HEPES, 11 glucose, and 0.025 EDTA, pH 7.4) supplemented with bovine serum albumin (5 g/100 ml). The aorta was tied off so that the venous effluent could be drained into a left atrial cannula. The heart and lungs (with a segment of the trachea) were removed en bloc from the thoracic cavity and mounted on a perfusion apparatus. The lung preparation was perfused via the pulmonary artery at a constant flow (0.035 ml/g), venous pressure (−4.0 cm H₂O), and temperature (37°C). A 50-ml volume of the perfusing liquid was recirculated. The perfusion apparatus was equipped with a pressure transducer and counter-weighted beam balance, which permitted continuous monitoring of pulmonary arterial pressure and lung weight during experiments. These data were displayed on a PC video monitor (IBM PS2 model 50z) after being digitized by an analog-to-digital converter (μDAS 8PGA board; Keithley Metrabyte, Solon, OH). Commercially available software (Notebook Pro for Windows; Labtech, Andover, MA) was used to control data acquisition and storage. In all experiments, the lung preparations underwent a 15-min equilibration perfusion. Use of preparations was isogravimetric and had a steady pulmonary arterial pressure (10−15 cm H₂O); the lungs were also kept inflated at a constant pressure (5 cm H₂O) throughout.

**EDTA experiments.** In experiments examining the effects of the chelator EDTA, lung preparations were equilibrated in a normal Krebs-Henseleit solution and then perfused for 20 min in an EDTA (5 mM)-Krebs (Ca concentration 2.4 mM) solution with a free ionized Ca concentration of ~10 μM (16). Determination of 125I-albumin permeability-surface area (PS) product and capillary filtration coefficient (K_{f,c}) was made as described in *Albumin PS product measurement and K_{f,c} measurement.*

*In situ cross-linking of gp60.* In experiments to test the effects of the gp60 cross-linking on the permeability of pulmonary microvessels, lung preparations underwent an equilibration perfusion. For cross-linking gp60, the perfusate received the anti-gp60 antibody for 5 min and then a secondary antibody (goat anti-rabbit IgG) for an additional 10 min. The antibodies were added directly to the perfusing liquid at a final concentration of 100 μg/ml. The control lung preparations received preimmune IgG. Determination of 125I-albumin PS product and K_{f,c} was made.

**Albumin PS product measurement.** Albumin PS product determinations were made by the "single-sample" technique of Kern et al. (15). Briefly, perfused lung preparations received 125I-albumin (~80,000 counts/ml) for 3 or 15 min (as indicated) followed by a 6-min washout with Krebs solution containing unlabeled albumin (5 g/100 ml) to remove cell surface and circulating 125I tracer. The lungs were detached from the perfusion apparatus and cut into 12 samples. Each sample was blotted, weighed, and counted for gamma radioactivity. Based on previous work by Kern and colleagues (15, 16), a tracer perfusion time of 3 min was sufficient for albumin to flux across the endothelium. Backflux of 125I-albumin from the pulmonary interstitium was considered negligible because of the brevity of the labeling period relative to the uptake time constant for albumin (~1 h) (15). Therefore, the uptake of 125I-albumin is taken as a unidirectional protein flux across the endothelial barrier and will predominantly represent tracer albumin that has been 1) transported via a transcellular pathway or 2) diffused via a paracellular pathway. PS product (in ml·min⁻¹·g dry lung⁻¹) was calculated with the formula A/(C_p·t), where A, C_p, and t are the tissue concentration of tracer albumin (in counts/g), the tracer concentration in the perfusing liquid (in counts/ml), and the exposure time, respectively.

**K_{f,c} measurement.** Determination of K_{f,c} was carried out in separate groups of lung preparations. After gp60 cross-linking or administration of preimmune IgG, K_{f,c} was measured from the rate of lung wet weight gain after a step increase (+6 cm H₂O) in venous pressure. The rate of weight gain was normalized by the lung wet weight and step size to calculate K_{f,c} in units of milliliters per minute per centimeters of water pressure. In experiments conducted with EDTA, K_{f,c} was determined 20 min after addition of the chelator when the lung wet weight rose to a new steady-state level. The analytic procedures used for computing K_{f,c} from recordings of lung wet weight are given in Swanson and Kern (34).

**Osmotic shock experiments.** Lung preparations were labeled with tracer albumin (~80,000 counts/ml) for 15 min. Vascular tracer was removed by a 6-min washout period. Perfusate samples after the 6-min rinse were free of tracer (relative to background level). We produced hypotonic shock by briefly (~15 s) flushing the pulmonary circuit with distilled H₂O. The distilled H₂O flush caused an immediate and precipitous rise in lung wet weight due to endothelial disruption. Next, we counted the total 125I-albumin tracer osmotically released into the venous effluent and the residual 125I-albumin tracer remaining in the lung tissue. The osmotically released albumin tracer was normalized by the dry weight of the lung tissue (counts/g). In some preparations, we also measured the osmotically released lactate dehydrogenase (LDH) in the venous effluent.

**LDH assay.** LDH activity in the effluent samples was determined with the Sigma Diagnostics lactase dehydrogenase kit for the quantitative, colorimetric determination of LDH. Results are expressed in Berger-Broida (B-B) units per milliliter and were read directly from the calibration curve [absorbance (525 nm) vs. LDH activity in B-B U/ml], where absorbance is directly proportional to the concentration of pyruvate substrate. One B-B unit is defined as that amount of LDH that will reduce 4.8 × 10⁻³ μmol of pyruvate to lactate per minute at 25°C.

**Influence of unlabeled albumin concentration on uptake of 125I-albumin.** In these experiments, the perfusing liquid contained labeled albumin of approximately constant activity (80,000 counts/ml) together with unlabeled albumin at concentrations between 0.05 and 5 g/100 ml. The 15-min 125I-albumin PS product was determined in each lung preparation.

**Statistical Analysis**

Statistical comparisons were made with Student’s *t*-test. The significance level was set at *P* < 0.05.

**RESULTS**

**Albumin Uptake and Transport in Rat Lung Microvessels**

**Albumin transfer through pulmonary microvessel walls in vivo.** Lung preparations received the perfusing liquid tracer albumin (~80,000 counts/ml) for 15 min. The vascular tracer was removed by a 6-min washout period. The wash liquid contained 5 g/100 ml of unlabeled albumin to displace any albumin tracer bound to the luminal endothelial cell surface of pulmonary vessels. Perfusate samples after the 6-min rinse were free of tracer (relative to the background level). We employed hypotonic shock to release any tracer that the vascular lining endothelial cells had internalized during the labeling period by briefly (~15 s) flushing the
pulmonary circuit with distilled H2O. The distilled H2O flush caused an immediate and precipitous rise in lung wet weight due to endothelial disruption. Separate experiments (see LDH release) confirmed that the distilled H2O treatment caused lysis of the endothelial cell layer. In each lung preparation, we counted the total 125I-albumin tracer osmotically released into the venous effluent and the residual 125I-albumin tracer remaining in the lung tissue. These counts, reported in Fig. 1A, were normalized by the dry weight of the lung preparation. The data (Fig. 1A) show that ~25% of the total tracer albumin taken up by the lung during the 15-min perfusion could be released by osmotic shock. The remaining counts were viewed as tracer albumin that crossed the endothelial barrier and thus were inaccessible to release by osmotic lysis of endothelial cells.

Filipin studies in vivo. We employed the sterol-binding agent filipin, an inhibitor of vesicular transcytosis, in the rat lung preparation to address whether 125I-albumin used an endocytic pathway to traverse the endothelium (31). The preparations underwent perfusion without and with filipin (300 ng/ml) for 20 min. The concentration of filipin employed did not affect the isogravimetric status of the lung preparations, implying that filipin exposure did not modify the barrier permeability to liquid. In one series of experiments, we determined the 15-min 125I-albumin PS product (see METHODS). Filipin (300 ng/ml) significantly reduced the 15-min 125I-albumin PS product from 11.8 ± 1.8 to 5.6 ± 0.8 µl·min⁻¹·g⁻¹ (Fig. 1B), indicating that ~50% of tracer albumin uptake into the lung tissue was inhibited. In a second series, we assessed the effects of filipin on the internalization of tracer albumin by the endothelium. Figure 1C shows the inhibitory effect of filipin (300 ng/ml) on the number of 125I-albumin counts recoverable in the effluent after osmotic shock to the pulmonary endothelium. Because filipin inhibited both osmotically releasable and residual tissue counts, the results indicate that filipin prevented uptake of tracer albumin in endothelial cells. However, the relative inhibitory effect of filipin was greater on the osmotically releasable counts than on the tissue counts (compare Fig. 1, B and C), suggesting that albumin transport through the pulmonary vessel wall was to some extent through a filipin-insensitive pathway.

LDH release. To verify that the osmotic release of 125I-albumin resulted from lysis of the endothelial cell layer, we made simultaneous measurements of LDH activity and tracer albumin levels in the effluent after a brief perfusion of the lung vessels with distilled H2O. To do this, an additional group of eight lung preparations was prelabeled for 15 min with 125I-albumin tracer and then subjected to hyposmotic shock as described in Albumin transfer through pulmonary microvessel walls in vivo. Four of the preparations also received filipin in the perfusate. The positive correlation that we found between the amount of tracer albumin released and the level of LDH activity measured in the same perfusate sample (Fig. 2) is evidence that previously internalized 125I-albumin was released after osmotic lysis of the vascular lining endothelium. Moreover, comparison of the two curves in Fig. 2 shows that for any chosen level of LDH activity, filipin reduced the number of osmotically releasable 125I-albumin counts by ~75%, thus confirming our finding that filipin reduced the internalization of tracer albumin in pulmonary vessel endothelial cells.

Activation of Albumin Uptake by gp60 in RPMECs

Cross-linking of gp60 with a polyclonal anti-gp60 antibody is a procedure used to induce clustering and activation of gp60 on the endothelial cell surface (35). To assess gp60 antibody as a tool for activating gp60 in the rat pulmonary microvascular endothelium, we studied the functional interaction of antibody with cultured RPMECs. We determined the effect of gp60 activation on the endocytosis of fluorescently labeled albumin. Confluent RPMEC monolayers seeded on
ruptured by brief distilled H2O flush (see METHODS). Flush liquid was removed during 6-min tracer washout period. Endothelium was labeled with tracer albumin (400,000 counts/ml) for 15 min. Vascular tracer effluent after osmotic shock of vascular endothelium. Lung preparations perfused with 300 ng/ml of filipin in the perfusate were labeled with tracer albumin. Cross-linking protocol for Krebs-perfused lungs was prepared with 10 µg/ml of anti-gp60 Ab or preimmune IgG for 30 min in cold (4°C) medium and washed with cold HBSS to remove unbound antibody. We permitted endocytosis of labeled albumin to proceed for 30 min by the introduction of warm HBSS (37°C) containing 50 µg/ml of Alexa 488-labeled albumin plus 0.5 mg/ml of unlabeled albumin. Unincorporated albumin fluorophore was removed by several cold rinses. Internalized tracer albumin (green) was visualized by confocal microscopy in optical planes passing about midway through the endothelial cell monolayer. Cell nuclei (blue) were identified by DAPI fluorescence. When internalized by endothelial cells, labeled albumin distributed into punctate inclusions (Fig. 3). We observed an appreciable constitutive uptake of fluorescent albumin in cells incubated with the control antibody (Fig. 3A). However, incubation with the anti-gp60 antibody produced a marked stimulation of uptake (Fig. 3B). Quantitative analysis (21) of cell fluorescence demonstrated that gp60 cross-linking produced an approximately twofold increase in the number of fluorescent inclusions compared with the control value.

The albumin-binding glycoprotein gp60 was found in cultured RPMECs with the Cy3-labeled anti-gp60 antibody (Fig. 4A). To investigate the possibility that gp60 is involved in the internalization of albumin by rat microvascular endothelial cells, we coincubated monolayer cultures with Alexa 488-labeled albumin and Cy3-labeled anti-gp60 antibody (see METHODS). The confocal images in Fig. 4, A and B, showed punctate intracellular distribution of Cy3 fluorescence and Alexa 488 fluorescence after a 15-min dye uptake period. In Fig. 4C, nearly complete colocalization of the Alexa 488 fluorescence (green image) and Cy3 fluorescence (red image) was evident by yellow in the merged image.

Because albumin appeared to incorporate into plasmalemmal vesicles containing gp60 as a membrane protein, we next used the styryl pyridinium dye RH 414 to mark for plasmalemmal vesicles in RPMECs by previously employed procedures (21). Colocalization of Alexa 488-labeled albumin and RH 414 in numerous small inclusions confirmed that albumin was internalized by endocytosis (Fig. 5A). However, experiments with a lysosome-marking fluorophore (LysoTracker) showed that the albumin did not enter the lysosomal compartment (Fig. 5B).

**Activation of Albumin Permeability by gp60**

Cross-linking in Rat Lung Microvessels

We carried out experiments using an isolated Krebs-perfused rat lung preparation to address the in vivo relevance of the cell culture findings. We first demonstrated by Western blotting the existence of immunoreactive gp60 in the rat lung (Fig. 6A). Fluorescence-activated cell sorter analysis of cultured RPMECs showed that rat lung endothelial cells expressed cell surface gp60 (Fig. 6B).

We measured the PS product for 125I-labeled albumin in Krebs-perfused rat lungs at 37°C to determine whether gp60 cross-linking in vivo increased albumin transport in intact pulmonary microvessels. The gp60 cross-linking protocol for Krebs-perfused lungs was
similar to that for endothelial monolayers (35); that is, anti-gp60 antibody (100 μg/ml) plus the secondary antibody (100 μg/ml) was added to the perfusing liquid as described in METHODS. Control lung preparations received only preimmune IgG (100 μg/ml). As shown in Fig. 7A, gp60 cross-linking increased 125I-albumin PS product by a factor of ~2.5 from the control values, indicating that activation of gp60 increased microvesSEL albumin permeability.

Because gp60 activation may increase the paracellular albumin transport pathway known to depend on the integrity of interendothelial cell junctions (8, 33), we next determined whether liquid permeability and albumin permeability in lung microvessels increased in parallel as the consequence of gp60 cross-linking. The following predictions can be made if the increase in albumin permeability occurs exclusively through the paracellular pathway: 1) increase in lung wet weight and 2) increased $K_{f,c}$, the measure of endothelial hydraulic conductivity. Figure 7B shows that EDTA (5.0 mM, 20 min) increased the permeability of the paracellular pathway in microvessels; that is, EDTA increased 125I-albumin PS product threefold, with associated increases in lung wet weight (Fig. 7C) and $K_{f,c}$ (Fig. 7D). In contrast, activation of gp60 by cross-linking had no significant effect on tissue fluid gain or $K_{f,c}$ (Fig. 7, C and D). Recordings of the continuous change in lung wet weight in the presence of EDTA or gp60 cross-linking reagents are shown in Fig. 8. Because the lungs were perfused at a constant flow and venous pressure and the pulmonary arterial pressure did not change during gp60 activation, the effect of gp60 cross-linking in selectively increasing albumin permeability did not result from increased capillary hydrostatic pressure. The finding that the increase in vessel wall 125I-albumin permeability occurred independently of increased liquid permeability indicates that albumin is transported across microvessels by a gp60-activated nonhydraulic and transcellular pathway.

If a portion of albumin transport through the vascular endothelium requires specific cell surface albumin-binding sites, the transport of tracer albumin should be reduced with increasing concentrations of unlabeled albumin (used as a competing ligand). The 15-min PS product for 125I-albumin was determined in a series of experiments in which a varying concentration of unlabeled albumin (from 0.05 to 5 g/100 ml) was used to compete with the 125I-labeled albumin. As shown in Fig. 9, the 125I-albumin PS product was decreased with unlabeled albumin concentrations > 1.0 g/100 ml (EC50 1.2 g/100 ml). For comparison, the effects of gp60 cross-linking (tested at unlabeled albumin concentrations of 3.2 and 5 g/100 ml) are given.

DISCUSSION

Tiruppathi et al. (35) originally purified the albumin-binding glycoprotein gp60 from bovine pulmonary microvessel endothelial cells. Immunocytochemical studies (20, 35) localized gp60 to the luminal surface of cultured microvascular endothelial cells. Antibodies raised against gp60 were shown to compete with the binding of native albumin to endothelial cell surface gp60 (36). Moreover, activation of gp60 in vitro by means of antibody cross-linking (enhanced with a secondary antibody) stimulated endocytosis and transcellular migration of proteins including fluorescent forms of anti-gp60 antibody and albumin (20, 36). We showed that gp60 activation induced two- to threefold increases in endothelial cell uptake and luminal-to-abluminal permeability of 125I-labeled tracer albumin and horseradish peroxidase (a fluid-phase tracer) (36).
These changes occurred without an increase in paracellular permeability as assessed by recording the transendothelial electrical impedance, a measure of interjunctional dimension in confluent endothelial cell monolayers (36).

In the present study, we addressed whether gp60 cross-linking can also activate albumin uptake in intact pulmonary microvessel endothelia and the transport of albumin across the microvessel barrier. After confirming that RPMECs possess cross-reactive antigen (Fig. 6), we showed that gp60 activation by antibody cross-linking (36) increased $^{125}$I-albumin PS product (a measure of vessel wall albumin permeability) two- to threefold without altering tissue fluid content or $K_{f,c}$ (i.e., vessel wall hydraulic conductivity). The increased albumin transport cannot be explained by increased capillary hydrostatic pressure and the resultant convective flux of albumin because 1) cross-linking has no effect on pulmonary arterial pressure and 2) venous pressure and perfusion rate were held constant during the experiment. We also performed separate experiments with EDTA, which induces interendothelial gap formation in microvessels through chelation of Ca$^{2+}$ and Mg$^{2+}$ (10, 11, 13, 17, 28), for comparison with the effects of gp60 activation. We observed in this experiment a marked and rapid rise in $^{125}$I-albumin PS product and an increase in tissue fluid content and $K_{f,c}$, reflecting increased paracellular permeability of both liquid and albumin. Therefore, we interpret the observed increase in $^{125}$I-albumin PS product in the absence of increased capillary hydrostatic pressure and liquid permeability after gp60 cross-linking to reflect activation of transport through a transcellular pathway. These results in pulmonary microvessels are consistent with the in vitro observation that gp60 cross-linking activates a transcellular, nonhydraulic pathway because the increase in albumin permeability was uncoupled from microvessel liquid permeability.

There are several explanations for the lack of increase in tissue fluid content in the face of increased vascular albumin permeability when pulmonary microvessel endothelia were subjected to gp60 cross-linking. One possibility is that the albumin transported into the interstitium via a transcellular pathway may be compartmentalized in the interstitial space such that it would not be osmotically active, whereas the albumin transported via interendothelial junctions can provide the oncotic pressure gradient needed for increased fluid filtration and accumulation. Several
groups (2, 3, 26) report that up to ~50% of the albumin in the interstitial space can be compartmentalized in an “excluded volume” where it remains osmotically inactive but slowly exchanges with the osmotically active interstitial albumin pool; thus albumin delivered to the basolateral endothelial surface via a transcellular pathway need not increase fluid filtration as seen in the present study. Another possibility based on the observation that gp60 remains bound to the exocytic vesicle membrane (20) is that interstitial albumin, by binding to gp60, may be shuttled back to the apical side, thus preventing accumulation of interstitial albumin and increasing the oncotic pressure.

We utilized cultured RPMECs to directly observe how the anti-gp60 Ab employed in the present study modified vesicle trafficking. We confirmed (Fig. 3) that gp60 cross-linking with an anti-gp60 antibody (against the bovine antigen) activated endocytic uptake of albumin in rat endothelial cells. Consistent with previous studies by Minshall et al. (20) and Tiruppathi et al. (36) on bovine or human endothelial cells, a fluorescent form of the anti-gp60 antibody strongly stained the cell surface of rat endothelial cells under cold conditions and was subsequently internalized (at 37°C) with albumin (Fig. 4) into punctate structures presumed to be endosomes. We demonstrated that this was indeed the case (Fig. 5) because fluoresently tagged albumin colocalized in the vesicles marked with the interfacial fluorescent probe RH 414 (21). These vesicles were not targeted to acidic cellular compartments such as lysosomes (Fig. 4) because LysoTracker did not colocalize with the internalized albumin fluorophore. The vesicles carrying albumin are likely to be involved in transcytosis because Minshall et al. (20) demonstrated that such vesicles also stained for gp60 and caveolin I and migrated to the basolateral cell surface where they released fluid-phase fluorescent markers such as FM 1-43 by exocytosis. In summary, the confocal images displayed in Figs. 3 and 4 clearly indicate that the anti-gp60 antibody used produced significant functional effects on rat endothelial cells.

In extending these in vitro findings to the intact rat lung preparation, we encountered the special problem of showing that tracer 125I-albumin actually crossed the endothelial barrier after a 15-min tracer uptake period at 37°C. We reasoned that tracer remaining in the lung after complete destruction of the endothelium would have crossed the endothelial barrier. Our evidence that osmotic shock (see METHODS) destroyed the endothelium is 1) the precipitous gain in lung wet weight accompanied by visible evidence of uniform and marked swelling of all lung lobes and 2) release of LDH into the effluent. We found that LDH release after osmotic shock was correlated with the release of tracer albumin (Fig. 2), suggesting that the two proteins were released together after lysis of the endothelium. Osmotic shock released ~25% of the iodinated albumin tracer taken up by lung tissue (Fig. 1). Because our data cannot establish whether osmotic shock destroyed all the endothelial cells in the pulmonary circulation, the figure of 25% must be interpreted as the minimum estimate of endothelium-associated counts. Arguably, the percentage of tracer albumin that crossed the endothelial barrier is at most 75% of the total tracer uptake into lung tissue.

We used filipin to probe the transport pathway in the intact lung. Filipin is a naturally occurring, lipophilic polyene antibiotic that binds to cholesterol in cell membranes and thereby perturbs the caveola structure and...
function (4, 22). Endothelial caveolae contain a rim of cross-linked cholesterol (32), and in the presence of filipin, caveolae disappear as distinct membrane structures within minutes (7). In agreement with the findings of others (31), we showed that 300 ng/ml of filipin applied for 20 min reduced the albumin PS product by 50% in the isolated rat lung preparation. We have extended these findings by showing that filipin also reduced (by 75%) osmotically releasable 125I-albumin counts, suggesting that the endothelium to some extent internalized tracer albumin in transit through the vascular endothelium. On the basis of these results, we attribute the overall reduction in the 125I-albumin PS product caused by filipin treatment to two factors, namely, reduction in transendothelial tracer flux and internalization of the tracer by the endothelium. These results and conclusions are seemingly at variance with those of Rippe and Taylor (29), who failed to find an expected increase in the albumin reflection coefficient in rat lung microvessels treated with filipin at concentrations of 0.22–1.8 μg/ml in the perfusate. It should be noted, however, that their baseline value for the albumin reflection coefficient was only 0.6–0.7, suggestive of substantial paracellular shunting of the iodinated albumin tracer used. Such shunting could have masked a filipin-sensitive transcellular tracer flux. Excess exposure to filipin (0.3 μg/ml) has been noted to increase extravasation of protein and fluid in the rat lung preparation (31) just as Rippe and Taylor (29) observed with relatively high concentrations of filipin (0.9 μg/ml). Presumably, this action of filipin represents a form of toxicity distinct from the inhibition of vesicle activity that occurs at filipin concentrations < 0.3 μg/ml.

If a portion of albumin transport through the endothelial cell layer requires a specific cell surface albumin-binding site, the transport of tracer albumin is predicted to decline with increasing concentrations of

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**Fig. 7.** A: gp60 cross-linking increases albumin transport in Krebs-perfused rat lungs. After a 15-min equilibration perfusion, cross-linking of gp60 was accomplished by sequential additions to perfusing liquid as follows: 1) anti-gp60 antibody (100 μg/ml) for 5 min and 2) secondary antibody (100 μg/ml) for 10 more min. At the 15th min, 125I-albumin PS product was determined by adding 125I-labeled albumin to perfusing liquid for 3 min followed by extensive rinsing to remove vascular label. B: after an equilibration perfusion (15 min), EDTA addition (5 mM for 20 min) caused a marked increase in the PS product for 125I-albumin through chelation of divalent cations (see METHODS). Chelation of divalent cations with EDTA (5.0 mM for 20 min) but not gp60 activation (15 min of gp60 cross-linking) increased lung wet weight (C) and capillary filtration coefficient (Kf,c; D) in lungs. Perfusion temperature was 37°C. Values are means ± SE; n = 3–4 preparations. *Significant difference from control.

**Fig. 8.** Original recordings of wet weight in isolated perfused rat lungs. Preparation remained isogravimetric during gp60 cross-linking (thick line). Chelation of divalent cations by EDTA caused lung wet weight to increase to a quasi steady state within 20 min, indicative of tissue fluid accumulation. Vascular pressures were stable throughout all experiments (data not shown). Additions (antibody or chelator) were made at 0 time. Note that records report changes in lung wet weight that was nulled at the start of lung perfusion (see METHODS).

**Fig. 9.** Effect of unlabeled albumin concentration ([Albumin], 0.05–5 g/100 ml) in the perfusate on 125I-albumin PS product. 125I-albumin concentration in the perfusate was constant at 80,000 counts/ml (see METHODS). Best-fitting theoretical function was drawn through the data points (IC50 of 1.2 g/100 ml; Hill coefficient of 4.0: maximum and minimum 125I-albumin PS products of 38 and 11 μl/min 1g−1, respectively; ○). For comparison, results with gp60-cross linking are given (△). Each point is representative of a different lung preparation. Note that data have been plotted on a semilogarithmic scale.
unlabeled albumin. The data reported in Fig. 9 confirm this prediction. The relationship between unlabeled albumin concentration and $^{125}\text{I}$-albumin PS product was sigmoidal. In the concentration range of 0.05–3.2 g/100 ml, unlabeled albumin reduced the PS product from 38 to 11 $\mu\text{g} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$, but further elevation of unlabeled albumin produced no additional effect, indicating that maximal displacement of tracer was reached. From these data, we have estimated that the unlabeled albumin inhibited tracer albumin uptake by $\sim 70\%$, with an IC$_{50}$ of 1.2 g/100 ml ($\sim 0.18 \text{mM}$). These results suggest that the majority of albumin tracer transported through the pulmonary microvessel wall was initially bound to low-affinity sites on the endothelial cell surface. It would be tempting to speculate that these sites contain gp60. However, in comparison with the present results, the binding affinity of albumin to gp60, expressed on the endothelial cell surface in vitro, was shown to be $\sim 100$-fold higher (36). Further investigation will be required to uncover the source of this discrepancy. The data in Fig. 9 also indicate that gp60 cross-linking modified the relationship between the unlabeled albumin concentration and the tracer albumin uptake, i.e., more tracer albumin was taken up at a given unlabeled albumin concentration. It is conceivable that gp60 cross-linking could have altered the affinity of vesicular carriers for albumin such that the content of albumin per vesicle was increased.

It is unlikely that raising the concentration of unlabeled albumin inhibited tracer uptake into the lung tissue because of a reduction in convection or an increase in perfusate oncotic pressure. First, all lung preparations regardless of unlabeled albumin concentration were in the isogravimetric condition before the addition of tracer albumin. Because isogravimetric preparations are in fluid balance, convective forces are theoretically negligible. Second, the $^{125}\text{I}$-albumin PS product was a constant function of unlabeled albumin in two concentration ranges (0.05–0.8 and 3.2–5 g/100 ml); the PS product showed a steep dependency on unlabeled albumin only in a very narrow concentration range centered about 1 g/100 ml. Hence the most marked changes in the PS product were associated with the least variation in oncotic pressure. Moreover, the oncotic pressure is a linear function of perfusate albumin concentration and therefore would not be expected to account for the nonlinear (hyperbolic) relationship between $^{125}\text{I}$-albumin PS product and unlabeled albumin concentration.

We have examined the in vivo relevance of transcytosis using fluorescence videomicroscopy of the vascular bed of the cremaster muscle in mice or rats to study the transfer of plasmalemmal vesicles (caveolae) across the microvessel barrier (37). RH 414 was used as a marker for vesicular traffic through endothelial cells. gp60 cross-linking with an anti-gp60 antibody stimulated the extravasation of RH 414 relative to FITC (used as a paracellular tracer), indicating that gp60 regulates vesicle trafficking in vivo. The present study in pulmonary microvessels has extended these earlier findings by showing that the vesicular carriers regulated by gp60 transport albumin through the microvessel wall. The tracer displacement study (Fig. 9) indicated that most of the albumin transferred through the microvessel wall is bound to a low-affinity site, probably associated with the vesicular carriers. Filipin apparently acts by disaggregating caveolae and thereby eliminating the albumin binding site and/or the vesicular carriers.

Among the activators of gp60 is albumin itself, a gp60 ligand, and antibody-induced cross-linking of gp60 (35). The activation of a transcellular pathway in endothelial cells capable of transporting albumin may serve as a mechanism for maintaining the interstitial oncotic pressure and hence in regulating tissue fluid balance. The activation of transcytosis in vascular endothelial cells by a strategy involving gp60 can be exploited as a means of delivering albumin-conjugated drugs and genes across the vascular endothelial barrier.

REFERENCES


