

## Reversibility of increased microvessel permeability in response to VE-cadherin disassembly

XIAOPEI GAO, PANOS KOUKLIS, NING XU, RICHARD D. MINSHALL,  
RAUDEL SANDOVAL, STEPHEN M. VOGEL, AND ASRAR B. MALIK

Department of Pharmacology, University of Illinois College of Medicine, Chicago, Illinois 60612

Received 8 June 2000; accepted in final form 23 June 2000

**Gao, Xiaopei, Panos Kouklis, Ning Xu, Richard D. Minshall, Raudel Sandoval, Stephen M. Vogel and Asrar B. Malik.** Reversibility of increased microvessel permeability in response to VE-cadherin disassembly. *Am J Physiol Lung Cell Mol Physiol* 279: L1218–L1225, 2000.—We determined the role of vascular endothelial (VE)-cadherin complex in regulating the permeability of pulmonary microvessels. Studies were made in mouse lungs perfused with albumin-Krebs containing EDTA, a  $\text{Ca}^{2+}$  chelator, added to study the VE-cadherin junctional disassembly. We then repleted the perfusate with  $\text{Ca}^{2+}$  to restore VE-cadherin integrity. Confocal microscopy showed a disappearance of VE-cadherin immunostaining in a time- and dose-dependent manner after  $\text{Ca}^{2+}$  chelation and reassembly of the VE-cadherin complex within 5 min after  $\text{Ca}^{2+}$  repletion. We determined the  $^{125}\text{I}$ -labeled albumin permeability-surface area product and capillary filtration coefficient ( $K_{fc}$ ) to quantify alterations in the pulmonary microvessel barrier. The addition of EDTA increased  $^{125}\text{I}$ -albumin permeability-surface area product and  $K_{fc}$  in a concentration-dependent manner within 5 min. The permeability response was reversed within 5 min after repletion of  $\text{Ca}^{2+}$ . An anti-VE-cadherin monoclonal antibody against epitopes responsible for homotypic adhesion augmented the increase in  $K_{fc}$  induced by  $\text{Ca}^{2+}$  chelation and prevented reversal of the response. We conclude that the disassembled VE-cadherins in endothelial cells are mobilized at the junctional plasmalemmal membrane such that VE-cadherins can rapidly form adhesive contact and restore microvessel permeability by reannealing the adherens junctions.

vascular endothelial-cadherin; homotypic adhesion; vascular permeability; ethylenediaminetetraacetic acid; isolated lungs; mouse

THE ENDOTHELIUM IS THE PRINCIPAL BARRIER to transvascular movement of proteins and circulating cells. Endothelial barrier function is highly dependent on specific adhesive molecules in interendothelial junctions and on the contractile state of the endothelial cells, which have the capacity to retract and subsequently form interendothelial gaps (9, 19–21, 31). Permeability-increasing agents such as thrombin and histamine can increase formation of interendothelial gaps by inducing an endothelial contractile force directed at interen-

dothelial junctions and decreasing intercellular adhesive forces (1, 11, 22, 32). The opening of interendothelial gaps occurs in part by the loss of integrity of the adherens junction complex. These junctions normally serve to maintain the semipermeable microvascular barrier and thus control the passage of liquid and solutes across the vessel wall. Although several interendothelial junctional structures may participate in the maintenance of the endothelial barrier (e.g., tight junctions and gap junctions), adherens junctions are particularly important because their function can be modulated by external stimuli (2, 6, 17, 24, 29). Studies have shown that integrity of the adherens junctions depends on extracellular  $\text{Ca}^{2+}$ , with a concentration of  $>1$  mM required to maintain homotypic adhesion of VE-cadherin pairs and thus a restrictive endothelial barrier (7, 18, 26). A reduction in perfusate  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  concentrations increased pulmonary vascular permeability and resulted in pulmonary edema (13, 15). Chelation of extracellular  $\text{Ca}^{2+}$  with EDTA was also accompanied by increased endothelial permeability in cultured endothelial monolayers in a time frame required for VE-cadherin redistribution (16, 25). VE-cadherins localized in the adherens junctions are the main endothelial adhesion proteins regulating cell-cell interactions and permeability of the microvascular endothelial barrier (6, 8, 20). VE-cadherins are members of a multiple gene family of cell adhesion molecules with a single-membrane-spanning domain and  $\text{Ca}^{2+}$ -dependent homophilic binding specificity (7, 12, 28). Although evidence suggests a role for VE-cadherins in regulating permeability of cultured endothelial monolayers, little is known about their function in intact microvessels. That VE-cadherin may play an important role in the microcirculation is evident in studies showing increased permeability of the microvessels of heart and lungs after treatment with the anti-VE-cadherin monoclonal antibody (MAb) VB-13 (5). Although the VE-cadherin-catenin complex can be disrupted subsequent to loss of homophilic adhesion, the question of reestablishing the junctional integrity is an important one for defining the physiological relevance of cadherin junctions in regulating endothelial barrier

Address for reprint requests and other correspondence: A. B. Malik, Dept. of Pharmacology, Univ. of Illinois College of Medicine, 835 South Wolcott Ave. (M/C 868), Chicago, IL 60612 (E-mail: abmalik@uic.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

function. It remains unclear whether loss of the VE-cadherin interaction is a rapidly reversible phenomenon. Hence, in the present study, we addressed the role of VE-cadherin-cadherin interaction in regulating junctional permeability and studied the reversibility of the microvessel permeability response in the intact mouse lung.

## METHODS

### *Ex Vivo Experimental Procedures*

**Lung preparation.** According to the approved protocol of the University of Illinois (Chicago, IL) Animal Care Committee, CD-1 male mice ( $n = 76$ ) weighing 30–35 g were placed in an anesthesia chamber and anesthetized with 3% halothane in room air at a flow rate of 2 l/min. After induction, anesthesia was continued by means of a nose cone. The trachea was cannulated with a polyethylene tube (PE-60; Becton Dickinson, Parsippany, NJ) for constant positive-pressure ventilation (rate of 186 breaths/min) with the anesthetic gas mixture. Heparin (50 U) was injected into the jugular vein to prevent blood clotting. The abdominal cavity was opened to expose the diaphragm that was ventrally punctured and cut free from the rib cage. Then, a thoracotomy was performed, and the two halves of the rib cage were retracted to expose the heart and lungs. To make the pulmonary artery accessible for cannulation, the heart was caudally retracted with a silk suture (6-0; Ethicon, Somerville, NJ) through the apical musculature. An incision was made in the right ventricle at the base of the pulmonary artery for the introduction of an arterial cannula, and another incision was made in the left atrium for drainage of venous effluent. In some preparations, a left atrial catheter was inserted. A polyethylene cannula (PE-60) was advanced into the pulmonary artery via the pulmonic valve and secured by means of a suture around the pulmonary artery that included the aorta. The lungs were perfused in situ with a peristaltic pump. The anesthetic gas flow was terminated when perfusion was begun, and ventilation was continued with room air. The heart and exsanguinated lungs were rapidly excised and transferred en bloc to a perfusion apparatus where lung preparations were suspended from a 6-cm Perspex lever arm attached to a force displacement transducer (FT03, Astro-Med, West Warwick, RI). The isolated lungs were ventilated (186 breaths/min) and perfused at a constant flow (2 ml/min), temperature (37°C), and venous pressure (0 cmH<sub>2</sub>O) with a modified Krebs-Henseleit solution (composition in mM: 118 NaCl, 4.7 KCl, 1.0 CaCl<sub>2</sub>, 0.5 MgCl<sub>2</sub>, 4.43 HEPES sodium, 5.57 HEPES, 3 NaHCO<sub>3</sub>, 11 glucose, and 0.025 EDTA, pH 7.4) supplemented with 5 g/100 ml of bovine serum albumin (BSA; fraction V, 99% pure, endotoxin free; Sigma-Aldrich, St. Louis, MO). Pulmonary arterial pressure was monitored throughout the experiment with a Gould pressure transducer (model P23ID, Gould Instruments, Dayton, OH). Lung wet weight was electronically nulled when the tissue was mounted, and subsequent weight changes due to gain or loss of fluid from the lung were recorded. Lung weight and arterial pressure recordings were displayed on a computer video monitor with the aid of amplifiers (model CP122, Astro-Med), an analog-to-digital converter (DAS 1800ST board; Keithly Metabyte, Solon, OH), and commercial software for acquisition and logging of data (Notebook Pro for Windows, Labtech, Andover, MA). All lung preparations underwent a 20-min equilibration perfusion. Lungs that were not isogravimetric at the end of the equilibration period were discarded.

**Capillary filtration coefficient measurement.** The capillary filtration coefficient ( $K_{fc}$ ) was measured to determine pulmonary microvascular permeability to liquid. Briefly, after the standard 20-min equilibration perfusion, the outflow pressure was rapidly elevated by 8 cmH<sub>2</sub>O for 3 min. The lung wet weight changed in a ramplike fashion, reflecting net fluid extravasation. At the end of each experiment, the lungs were dissected free of nonpulmonary tissue, and lung dry weight was determined.  $K_{fc}$  was calculated from the slope of the recorded weight change normalized to the pressure change and the lung dry weight. The wet-to-dry weight ratio in six freshly isolated (nonperfused) mouse lungs was  $6.04 \pm 0.4$ .

**<sup>125</sup>I-albumin permeability-surface area product measurement.** BSA was labeled with <sup>125</sup>I (New England Nuclear, Boston, MA) with the chloramine T method (3). Free <sup>125</sup>I was separated from <sup>125</sup>I-BSA with a Sephadex G25 column; free <sup>125</sup>I contributed <0.3% of the total radioactivity as determined by trichloroacetic acid precipitation. Albumin-Krebs solution containing <sup>125</sup>I-labeled BSA (~2,000,000 counts/ml) was infused at 0.2 ml/min (model sp100i syringe pump, World Precision Instruments, Sarasota, FL) into the pulmonary artery for 3 min. A sample of the venous effluent was saved for later determination of the perfusate tracer concentration (in counts/ml). Vascular tracer was removed during a 6-min washout period with unlabeled albumin-Krebs solution as previously described (27). Then, the lung tissue and the previously collected venous effluent sample were counted for <sup>125</sup>I in a gamma counter (Minaxi Auto Gamma 5000 series gamma counter, Packard Instruments, Downers Grove, IL). The lung tissue was dried to a constant weight in an oven at 80°C. The <sup>125</sup>I-albumin permeability-surface area ( $PS$ ) product was calculated from  $A/(C \times t)$ , where  $A$  is measured tissue counts per gram of dry lung tissue,  $t$  is duration of exposure to tracer BSA (in min), and  $C$  is concentration of <sup>125</sup>I-BSA in the perfusing liquid (14).

**Histochemistry.** ANTIBODIES. Rat anti-mouse MAb against VE-cadherin (clone 11D4.1) was purchased from RDI Research Diagnostics (Flanders, NJ). Rabbit anti-human von Willebrand factor polyclonal antibody (Ab) was purchased from Sigma-Aldrich. Texas Red dye-conjugated AffiniPure F(ab')<sub>2</sub> fragment anti-rat IgG (H+L) was purchased from Jackson ImmunoResearch (West Grove, PA). Alexa 488 goat anti-rabbit IgG was purchased from Molecular Probes (Eugene, OR).

**FROZEN SECTIONS.** After detachment from the perfusion apparatus, lung tissues were immediately treated with optimum cutting temperature embedding compound (Miles Diagnostics, Elkhart, IN) and frozen in a small vessel containing 2-methylbutane (liquid isopentane) precooled to about -70°C in liquid nitrogen. Sections were cut at 7 μm and then double stained with anti-VE-cadherin MAb and anti-von Willebrand factor Ab according to the following protocol. Sections were fixed in methanol (HPLC grade) for 10 min at -20°C. A blocking solution of 2% BSA and 5% goat serum in PBS was applied for 20 min followed by rinsing three times for 5 min each in PBS. The primary Abs, anti-VE-cadherin MAb (clone 11D4.1, diluted 1:600 in blocking solution) and anti-von Willebrand factor Ab (factor VIII, diluted 1:1,600 in blocking solution), were applied for 1 h at room temperature. Sections were rinsed three times for 5 min each in PBS and then incubated for 45 min with Texas Red-labeled anti-rat secondary Ab (1:200 dilution) or Alexa 488-labeled anti-rabbit secondary Ab (1:500 dilution) in blocking solution. The slides were then washed three times for 5 min each and mounted on glass coverslips for examination by confocal microscopy. Nonspecific immunofluorescence of the secondary Abs used was determined by substituting



rat IgG or rabbit IgG for the primary Abs and then staining the sections with the secondary Ab.

**CONFOCAL MICROSCOPY.** A laser (Ar-Kr)-scanning confocal microscope (Zeiss LSM 510) was used for viewing fluorescence of mouse lung sections in chosen optical planes. The section plane was advanced in 0.2- $\mu$ m increments through the specimen. At each optical plane, the specimen was scanned at 488 nm to excite FITC and/or at 568 nm to excite Texas Red.

### *In Vitro Experimental Procedures*

**Endothelial cell cultures.** Confluent monolayers of human endothelial cells from the umbilical vein (HUVECs) were obtained from VEC Technologies (Rensselaer, NY). The cells were grown in EBM-2 medium supplemented with 10% fetal bovine serum. HUVECs passaged between three and seven times were used for all experiments.

**Endothelial monolayer resistance measurements.** Experiments were carried out according to previously detailed procedures (30). Briefly, HUVECs were seeded on gelatin-coated gold electrodes ( $4.9 \times 10^{-4}$  cm<sup>2</sup>) and grown to confluence. The small electrode and a larger counterelectrode were connected to a phase-sensitive lock-in amplifier. A constant current ( $\sim 1$   $\mu$ A) was supplied by connecting a sinusoidal voltage source (1 V, 4,000 Hz) to a 1-M $\Omega$  resistor in series with the small electrode and the larger counterelectrode. The resulting voltage that developed between the small electrode and larger counterelectrode was acquired continuously with a personal computer and used to calculate monolayer resistance values on-line. The same computer also controlled the output of the amplifier and switched the measurement to different electrodes during the course of an experiment. Before the experiments, confluent endothelial monolayers were incubated in DMEM containing 10 mM HEPES, pH 7.4, for 0.5 h, and then the EDTA-induced changes in resistance of the endothelial monolayer were measured. The data are presented as a change in resistance normalized to its value at time 0 as previously described (10).

**VE-cadherin immunostaining of endothelial monolayers.** Endothelial cells were grown to confluence on glass coverslips coated with 0.1% gelatin. The cells were washed twice with Hanks' balanced salt solution (HBSS) and fixed with 4% paraformaldehyde in HBSS for 20 min at 25°C. The cells were then washed three times with 0.1 M glycine in HBSS for 5 min and three times with HBSS for 10 min followed by incubation with blocking buffer (HBSS containing 5% goat serum, 0.2% BSA, 0.01% sodium azide, and 0.1% Triton X-100) at 25°C for 1 h. The blocking buffer was removed, and the cells were incubated at 25°C for 2 h with 2  $\mu$ g/ml of mouse anti-VE-cadherin MAb in blocking buffer. After incubation, the cells were washed three times with HBSS and incubated with Alexa 488 goat anti-mouse IgG in blocking buffer at 25°C for 1 h. The cells were then washed three times with HBSS. The coverslips were mounted onto glass slides containing ProLong Antifade mounting medium and allowed to dry at room temperature overnight.

**Digital fluorescence microscopy.** Fluorescent imaging of endothelial cell monolayers immunostained for VE-cadherin was performed with an inverted Nikon microscope according to published procedures (23). For fluorescence observation, the dye in the cells was excited by a 100-W mercury arc lamp. The excitation wavelength was selected with filter cubes appropriate for FITC fluorescence. Fluorescence and differential interference contrast images were recorded for each cell field with a cooled integrating charge-coupled device camera (Imagepoint, Photometrics, Phoenix, AZ).

### *Experimental Protocols for Mouse Lung Preparations*

**Effects of Ca<sup>2+</sup> chelation on lung wet weight.** Lung preparations were equilibrated for 20 min by perfusion with 5% albumin in Krebs buffer to establish isogravimetric conditions. To induce Ca<sup>2+</sup> chelation, EDTA was infused into the pulmonary circulation at 0.2 ml/min for 20 min, achieving final perfusate EDTA concentrations of 0.5, 1.0, 2.0, or 2.5 mM (one concentration was used per lung preparation). EDTA administration was followed by a washout period of up to 60 min to replete perfusate Ca<sup>2+</sup> and reverse the effects of EDTA. Lung wet weight and pulmonary arterial pressure were continuously monitored in each of the groups ( $n = 4-5$  lungs/group).

**Influence of Ca<sup>2+</sup> chelation on <sup>125</sup>I-albumin PS product.** We determined the changes in pulmonary microvascular protein permeability in mice after Ca<sup>2+</sup> chelation using EDTA added to the perfusate. Preparations were first treated with EDTA (0.5, 1.0, or 2.0 mM) for a period of 20 min. Then, the <sup>125</sup>I-BSA PS product was determined (see <sup>125</sup>I-albumin permeability-surface area product measurement). Each group contained four to five lungs.

**Influence of Ca<sup>2+</sup> chelation on K<sub>fc</sub>.** We determined the lung transvascular water permeability induced by Ca<sup>2+</sup> chelation and the reversibility of this effect. After the 20-min equilibration period, we determined a baseline K<sub>fc</sub> value. In different groups of lung preparations, 0, 0.5, 1.0, or 2.0 mM EDTA was infused into the pulmonary circulation (0.2 ml/min) for 20 min followed by a 60-min washout period. K<sub>fc</sub> was measured at various times during and after perfusion with EDTA. Each group contained four to five lungs.

**Effect of anti-VE-cadherin MAb on pulmonary microvascular liquid permeability.** We determined the effects of a MAb directed against an extramembranous epitope of mouse VE-cadherin (clone 11D4.1) on the increase in lung microvascular liquid permeability induced by Ca<sup>2+</sup> chelation and the reversibility of this effect. After a 20-min equilibration period, a baseline K<sub>fc</sub> measurement was made. Anti-VE-cadherin MAb (100  $\mu$ g/ml) was added to the recirculating perfusate for 30 min. Then, 2.5 mM EDTA was added to the perfusate for 5-7 min, and a test measurement of K<sub>fc</sub> was made. EDTA was removed to replete buffer Ca<sup>2+</sup> concentration, and a final K<sub>fc</sub> measurement was made. Each group contained four to five lungs. In additional experiments, the influence of anti-cadherin MAb in the absence of EDTA was also determined. In these experiments, lung preparations were exposed to the MAb for 60 min, during which measurements of K<sub>fc</sub> were made; control experiments were carried out in an identical fashion except for omission of the MAb or substitution with rat IgG. Each group consisted of four to five lung preparations.

**Statistical analysis.** Data are expressed as means  $\pm$  SE. Statistical analysis was performed with two-way analysis of variance and Newman-Keuls test for multiple comparisons. The number of experiments is given in Figs. 1-5. A value of  $P < 0.05$  was the criterion for significance.

## RESULTS

### *Immunostaining of VE-Cadherin Disassembly and Its Reversal*

Figure 1 shows staining of mouse lung microvessels for VE-cadherin before and after junctional disaggregation by EDTA treatment. Lungs perfused for 5, 15, and 30 min with 2.0 mM EDTA showed a loss of VE-cadherin staining; the change in staining pattern

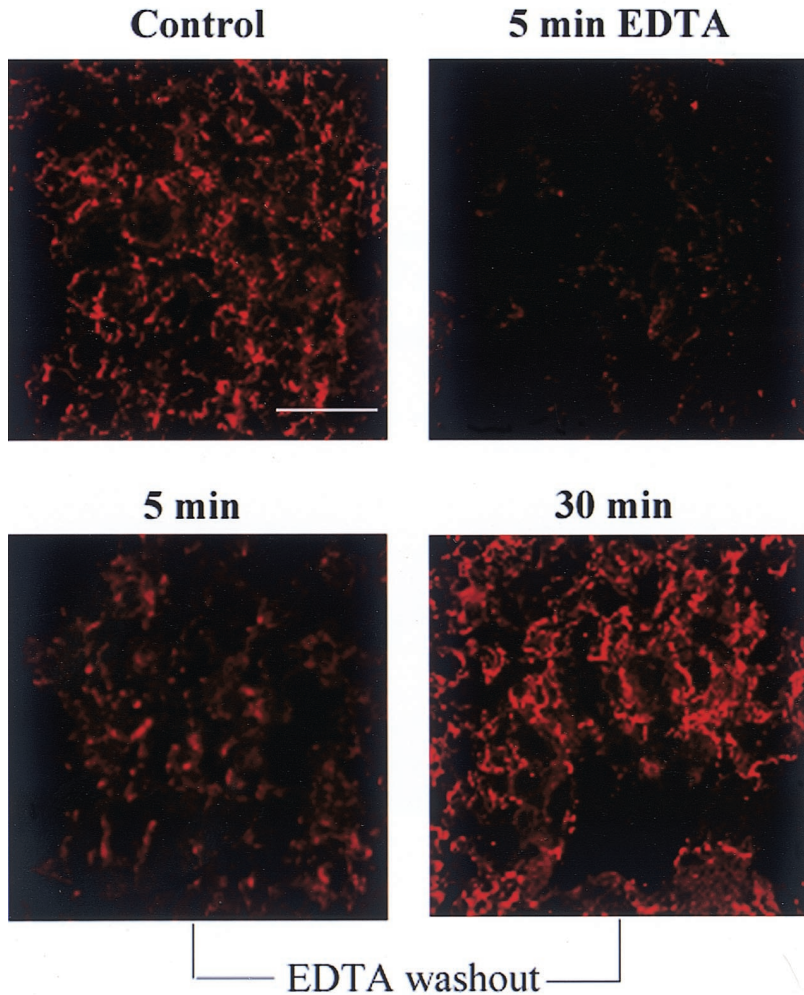


Fig. 1. Confocal images of alveolar-capillary septa in mouse lungs after staining with anti-vascular endothelial (VE)-cadherin monoclonal antibody (MAb) that binds to an extramembranous epitope (described in METHODS). Results are typical of 4 experiments. Results show the virtual loss of VE-cadherin staining after EDTA treatment (at 5 min). Results also show reappearance of staining within 5 min after repletion of  $\text{Ca}^{2+}$  in the perfusate and a complete recovery within 30 min. Bar, 40  $\mu\text{m}$ .

at 5 min is shown in Fig. 1. Removal of the  $\text{Ca}^{2+}$  chelator from the perfusate resulted in the reappearance of junctional VE-cadherin staining, beginning <5 min after  $\text{Ca}^{2+}$  repletion and progressing to a full restoration of staining within 30 min (Fig. 1).

#### *VE-Cadherin Disassembly Increases Pulmonary Microvessel Permeability and Lung Weight*

Results of  $K_{fc}$  and  $^{125}\text{I}$ -albumin  $PS$  product measurements are shown in Fig. 2.  $K_{fc}$  showed a time- and concentration-dependent increase (Fig. 2A). The increases in liquid permeability in response to EDTA paralleled those in the  $^{125}\text{I}$ -albumin  $PS$  product (Fig. 2B).  $^{125}\text{I}$ -albumin  $PS$  product (measured after 20 min in EDTA) increased significantly in response to 1.0 mM EDTA and was increased further with 2.0 mM EDTA (Fig. 2B). No significant change in  $^{125}\text{I}$ -albumin  $PS$  was observed with the addition of 0.5 mM EDTA (Fig. 2B).

Perfusion of the microvessels with EDTA (2.0 mM) for 20 min resulted in marked increases in lung wet weight (Fig. 2C). The increase occurred with a latency of ~16 min during the addition of EDTA. After removal of EDTA, lung weight increased thereafter to a value 77% above baseline 70 min after the addition of EDTA. Pulmonary arterial pressure remained unchanged dur-

ing the period of EDTA application (Table 1) and remained stable thereafter. EDTA at a concentration of 0.5 mM did not significantly affect lung wet weight (Fig. 2C). EDTA at 1.0 mM produced a small but significant increase in lung wet weight above the control value (Fig. 2C). Infusion of 5% albumin-Krebs containing no EDTA for 20 min had no effect on lung weight and pulmonary arterial pressure (Fig. 2C).

#### *Reversibility of Increased Pulmonary Microvessel Permeability After VE-Cadherin Reassembly*

We observed that repletion of  $\text{Ca}^{2+}$  resulted in a rapid reversal of the increased  $K_{fc}$  induced by  $\text{Ca}^{2+}$  chelation. The reversal occurred after multiple concentrations of EDTA (1–2.5 mM) were used to disrupt the junctions. Reversibility of the effect of 5- to 7-min applications of 2.5 mM EDTA was complete within 20 min of restoration of normal perfusate  $\text{Ca}^{2+}$  concentration (Fig. 3A). Interestingly, the addition of anti-VE-cadherin MAb prevented the reversibility of the EDTA-induced rise in  $K_{fc}$  (Fig. 3A). In addition, the anti-VE-cadherin MAb augmented the increase in  $K_{fc}$  induced by removal of  $\text{Ca}^{2+}$  from the perfusate (Fig. 3A). In contrast, rat IgG, used in control experiments, had no significant effect on the EDTA-induced increase in

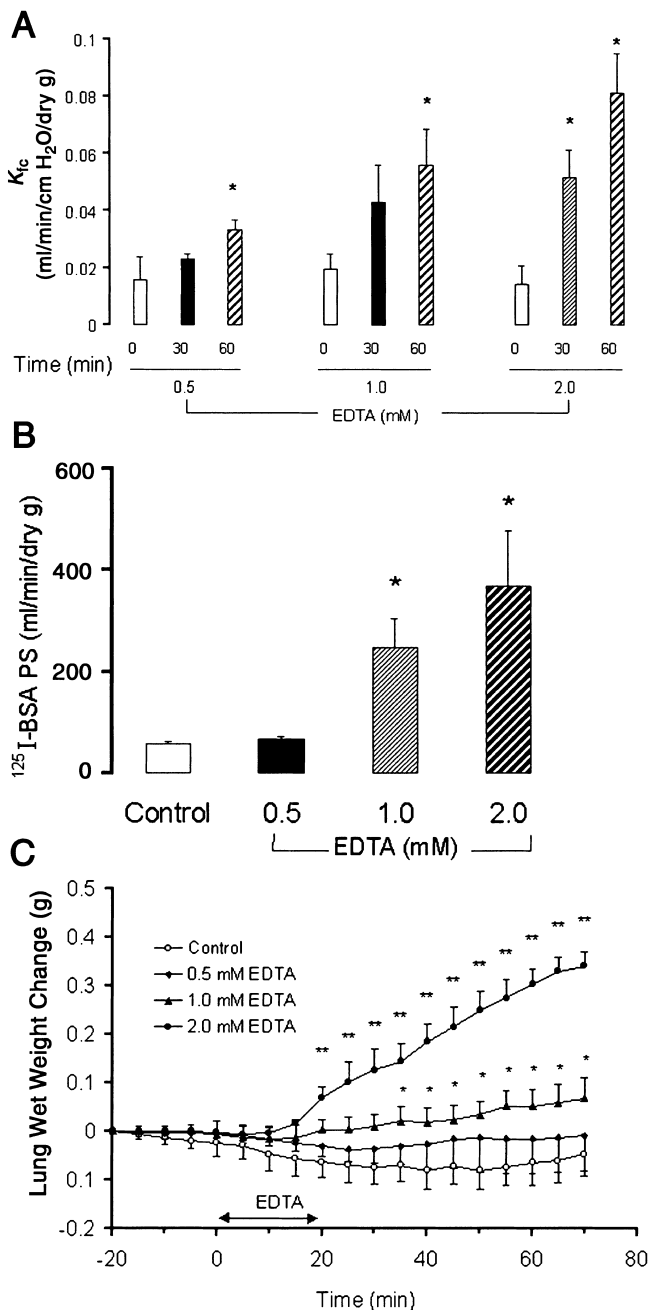


Fig. 2. A: effect of EDTA on capillary filtration coefficient ( $K_{fc}$ ) in isolated-perfused mouse lung preparations.  $K_{fc}$  measurements were made 0, 30, or 60 min after lung equilibration (see METHODS). EDTA at the indicated concentrations was applied for 20 min after baseline measurement (0-time values). Values are means  $\pm$  SE;  $n = 4-5$  lung preparations/group. \*Significant increase over control value,  $P < 0.05$ . B: effect of EDTA on  $^{125}\text{I}$ -labeled BSA permeability-surface area (PS) product. EDTA at the indicated concentrations was applied for 20 min, and then the 3-min  $^{125}\text{I}$ -BSA PS product was determined. Values are means  $\pm$  SE;  $n = 4-5$  lung preparations/group. Note significant increases in PS product at EDTA concentrations of 1.0 and 2.0 mM. C: EDTA induction of pulmonary edema as measured by the increase in lung wet weight. EDTA was added to perfusing liquid at the indicated time and concentrations while lung wet weight was monitored. Values are means  $\pm$  SE;  $n = 4-5$  lung preparations/group. \*Significant EDTA (1.0 mM)-induced increase in  $K_{fc}$  over baseline and control values. \*\*Significant effect of EDTA (2.0 mM) over baseline and control values.

Table 1. Lack of effect of EDTA on pulmonary perfusion pressure in isolated perfused mouse lung preparations

[EDTA], mM	Perfusion Pressure, cmH <sub>2</sub> O	
	Basal	EDTA
0.5	7.0 $\pm$ 0.4	7.3 $\pm$ 0.6
1.0	7.2 $\pm$ 0.5	7.2 $\pm$ 0.7
2.0	7.4 $\pm$ 0.8	7.3 $\pm$ 0.5

Values are means  $\pm$  SE. [EDTA], EDTA concentration. Values were obtained in the presence of EDTA after a 20-min treatment period.

$K_{fc}$  or on its reversibility after  $\text{Ca}^{2+}$  repletion (Fig. 3A). The anti-VE-cadherin MAb alone produced a small but significant increase in  $K_{fc}$  compared with that in untreated lung preparations (Fig. 3B).

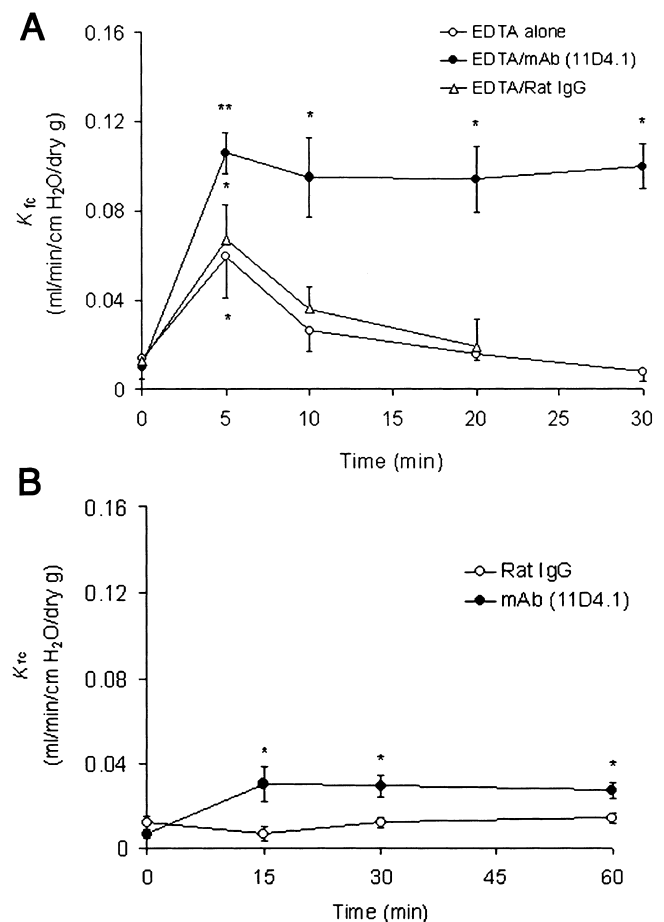


Fig. 3. A: reversible increase in  $K_{fc}$  induced by EDTA and potentiating effect of an anti-VE-cadherin MAb. Rat IgG or anti-VE-cadherin MAb was administered at a perfusate concentration of 100  $\mu\text{g/ml}$  30 min before EDTA addition. EDTA was administered in the perfusate from 0 to 5 min and then removed. Values are means  $\pm$  SE;  $n = 4$  preparations/group. Note the reversible effect on  $K_{fc}$  of EDTA alone (2.5 mM) or of EDTA in the presence of control IgG. Also note that the MAb directed against an extramembranous VE-cadherin epitope increased the peak effect of EDTA and prevented the reversibility of EDTA. \*Significant increase compared with baseline value. \*\*Significant potentiation by MAb over 5-min control value. B: influence of anti-VE-cadherin MAb (100  $\mu\text{g/ml}$ ) on  $K_{fc}$  compared with control rat IgG (100  $\mu\text{g/ml}$ ;  $n = 4$  preparations/group). \*Significant increase in  $K_{fc}$  over control value.



*Loss of VE-Cadherin Junctional Assembly and Its Reversibility in Endothelial Monolayers Is Associated With a Reversible Decrease in Transendothelial Electrical Resistance*

We used endothelial monolayers to address the redistribution of VE-cadherins during and after adherens junctional disassembly induced by EDTA and its relationship to monolayer permeability as determined by real-time measurement of monolayer electrical resistance. EDTA applied for 20 min produced a concentration-dependent decrease in monolayer resistance (Fig. 4A). Removal of EDTA (i.e., repletion of  $\text{Ca}^{2+}$ ) caused the transendothelial resistance to rise in proportion to the amount of EDTA used to disassemble the junctions in Fig. 4A (Fig. 4B).

EDTA (2.0 mM) exposure for 5 min or longer caused a complete disappearance of junctional VE-cadherin immunostaining in endothelial monolayers. Diffuse intracellular staining of VE-cadherin (Fig. 5) could be detected under these conditions. Repletion of  $\text{Ca}^{2+}$  by

removal of EDTA caused a reappearance of VE-cadherin immunostaining (Fig. 5).

## DISCUSSION

In the present study, we addressed the effects of the loss of VE-cadherin junctional assembly on microvessel endothelial permeability and the reversal of the loss of barrier function after VE-cadherin junctions were reannealed in the intact mouse lung. We observed that EDTA added to the perfusate increased pulmonary microvascular permeability. A pronounced increase in  $K_{fc}$  occurred within 5–7 min of EDTA (2.5 mM) application and was followed some 10–15 min later by barrier breakdown, signified by marked elevations in lung wet weight and  $^{125}\text{I}$ -BSA PS product. We observed that the EDTA-induced rise in  $K_{fc}$  was readily reversible as long as EDTA exposure was kept brief enough to avoid frank edema. Thus the perfused mouse lung was shown to be a valid experimental preparation in which the cycle of junctional disassembly and reassembly could be studied. Moreover, the preparation lent itself to correlational immunocytochemical analysis.

To pursue these studies, it was first important to establish the baseline stability of the mouse lung preparation. To do this, we continuously monitored lung wet weight and pulmonary arterial pressure for a 120-min period, and the results showed that the mouse lung remained stable; i.e., the preparations did not gain weight during this period of perfusion and lung ventilation and the perfusion pressure was constant. Typical preparations actually showed a slight weight decrease, probably secondary to evaporative loss of fluid from the lung surface. The stability of the mouse lung preparation makes it a useful model for assessment of microvascular permeability in the intact lung.

The increased  $K_{fc}$  in  $\text{Ca}^{2+}$ -depleted lung preparations was induced by loss of adherens junctional contacts as evidenced by decreased VE-cadherin immunostaining at the sites of cell-cell junctions. Junctional assembly was reestablished in functional terms within 20 min by restoration of  $\text{Ca}^{2+}$  to the perfusate and was associated with morphological reestablishment of the VE-cadherin junctions. A MAbs directed against the extramembranous homotypic domain of VE-cadherin prevented the reversibility of the permeability increase. These results indicate that loss of cadherin-cadherin interaction induced the increase in junctional endothelial permeability and that the response was rapidly reversible. Because junctional reannealing occurred rapidly in intact microvessels, the results suggest that the components of the disassembled cadherin junctional complex remained intact and could be reassembled within minutes. Immunostaining in endothelial cell monolayers revealed that there was a loss of cell surface VE-cadherin staining when the junctions were disrupted with EDTA. The morphological and functional recovery of the disaggregated junctions in endothelial monolayers suggests that intracellularly

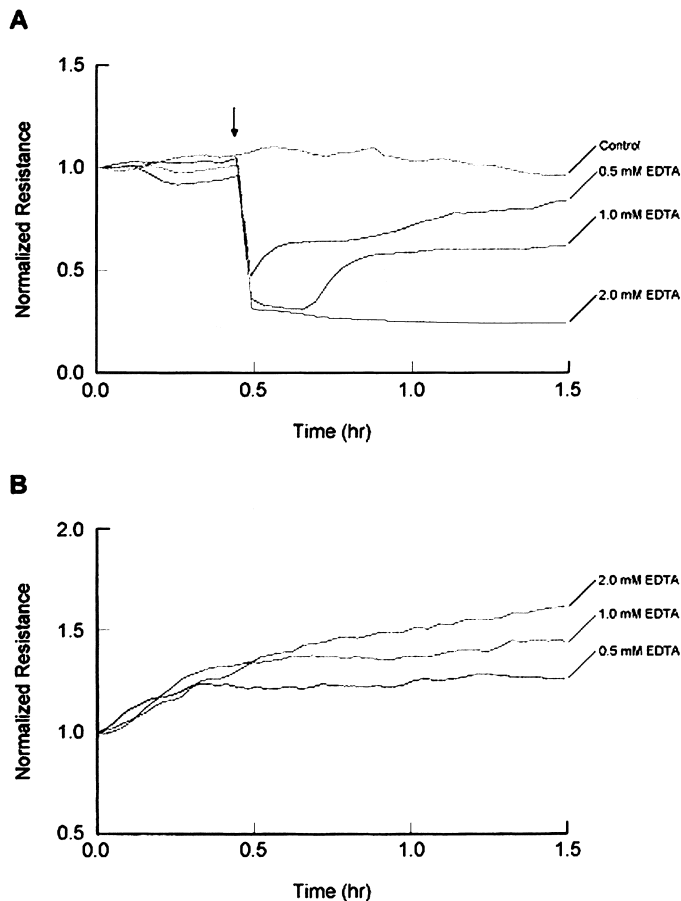
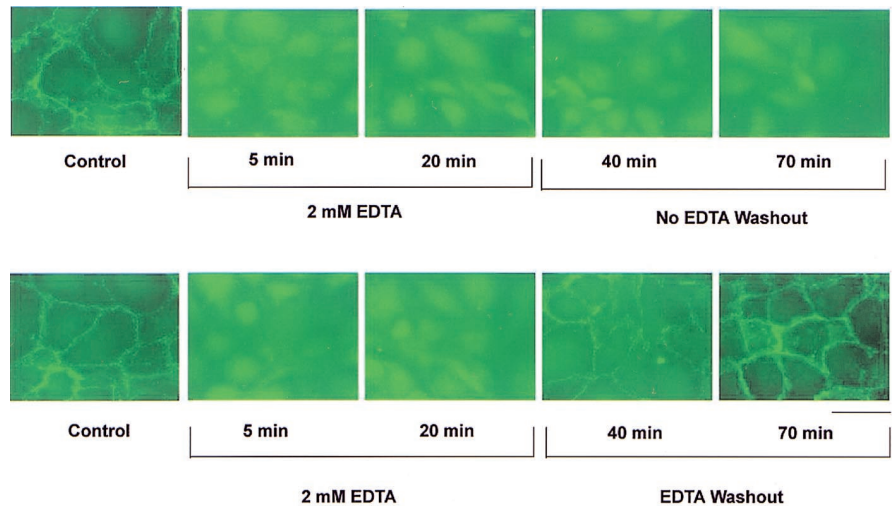


Fig. 4. A: influence of EDTA on transendothelial electrical resistance of human umbilical vein endothelial cell (HUVEC) monolayers. Resistance-lowering effects of EDTA at indicated concentrations (arrow) are seen in plots of normalized monolayer resistance vs. time. B: recovery time course of monolayer resistance by washout of EDTA; chelator was applied for 30 min. Results indicate recovery of electrical resistance within 30 min after restoration of normal  $\text{Ca}^{2+}$  concentration. Results are typical of 4 experiments.

Fig. 5. Digital fluorescence micrographs showing disappearance and reappearance of VE-cadherin immunostaining in HUVEC monolayers as a result of  $\text{Ca}^{2+}$  depletion and repletion. Rapid loss of VE-cadherin staining (see METHODS) was observed within 5 min after addition of EDTA and was persistent; restoration in staining was partial within 40 min after repletion of  $\text{Ca}^{2+}$  and complete by 70 min. Time course of recovery was consistent with restoration of electrical resistance (Fig. 4). Results are typical of 3 experiments. Bar, 25  $\mu\text{m}$ .



sequestered VE-cadherin could be recruited to the cell junctions during restoration of adherens junctions.

A proposal has been made that the increased vascular endothelial permeability in response to inflammatory mediators is the result of release of tensile forces generated at the sites of cell-cell and cell-extracellular matrix adhesion (22). Implicit in this concept is that endothelial cells are held in a state of tension such that the increased paracellular permeability occurs by the temporary loss of junctional integrity or alterations in contact between endothelial cell and extracellular matrix binding sites (such as at the focal adhesion complex) (22). The present results showing that  $\text{Ca}^{2+}$  chelation resulted in rapid VE-cadherin disassembly *in vivo*, loss of cell-cell interaction (as demonstrated by decreased transendothelial resistance), and increased pulmonary microvascular permeability to liquid and albumin support this hypothesis as a mechanism mediating increased microvascular endothelial permeability. Thus our results support the concept that VE-cadherin is a key regulator of endothelial cell permeability. An important finding is that the increase in microvascular permeability can be dynamically regulated in that the response is rapidly reversible. This finding suggests that uncoupled VE-cadherins are sequestered in the cell so that the junctions can be rapidly reannealed on activation by appropriate stimuli. The reversibility of the response does not support the suggestion that there is uncoupling of the cadherins and catenins and internalization of the components of this complex (33) leading to loss of junctional integrity.

In the present study, the increase in pulmonary microvascular permeability and its reversal induced by uncoupling and then recoupling of VE-cadherins was associated with characteristic morphological alterations at the level of interendothelial junctions (16). We observed that  $\text{Ca}^{2+}$  chelation resulted in decreased VE-cadherin immunostaining and that this was associated with increased microvascular  $^{125}\text{I}$ -albumin permeability and  $K_{fc}$ . In addition, reversal of the increased permeability induced by repleting the perfusate  $\text{Ca}^{2+}$  was associated with the re-forming of the adherens

junctions and increased VE-cadherin immunostaining at the junctions. Thus changes in microvessel permeability can be ascribed directly to alterations in VE-cadherin junctions. Similar observations were made in cultured endothelial cells in which the loss of VE-cadherin staining was coupled to a decrease in transendothelial electrical resistance and the resistance response was reversed with the reassembly of VE-cadherins.

We considered whether the opening and closing of endothelial junctions could be regulated by redistribution of VE-cadherins. Because  $\text{Ca}^{2+}$  is required for maintenance of the functional conformation of VE-cadherins, which is essential for junctional integrity (4), we used an anti-VE-cadherin MAb added to the perfusate to test the concept that the VE-cadherins are redistributed after removal of  $\text{Ca}^{2+}$  from the perfusate. Pretreatment with the VE-cadherin MAb significantly increased the EDTA-induced lung wet weight gain and  $K_{fc}$ , and, importantly, it prevented the rapid reversal of the increase in capillary permeability on restoring the  $\text{Ca}^{2+}$  concentration. We observed that the anti-VE cadherin MAb *per se* produced only a marginal increase in liquid permeability, suggesting that the epitope was protected from access to the MAb until junctional complexes were dissociated. Brief EDTA exposure that disaggregated junctional complexes arguably could have exposed the binding epitope and allowed the MAb to bind, thus potentiating the permeability-enhancing effect of EDTA and preventing the reversibility of EDTA. These findings are consistent with the observation that intravenous administration of anti-VE-cadherin Ab increased lung microvascular permeability during a more sustained application of 2 h in the sense that unchallenged adherens junctional complexes should be stable relative to those challenged with EDTA (5). Because the anti-VE-cadherin MAb prevented the reversibility of the response, our data suggest that uncoupling of VE-cadherins results in sequestration of VE-cadherin and perhaps other macromolecular constituents of adherens junctions in or near the plasmalemmal membrane so that VE-cadherins can be readily rejoined on restoring  $\text{Ca}^{2+}$  concentration.

We thank Dr. Meiling Chen for providing expert assistance in the acquisition and interpretation of confocal images. We gratefully acknowledge Dr. M. Sakosan for many valuable discussions regarding the manuscript.

This study was supported by National Heart, Lung, and Blood Institute Grants HL-45638, HL-60678, and HL-46350.

## REFERENCES

- Baldwin AL and Thurston G. Changes in endothelial actin cytoskeleton in venules with time after histamine treatment. *Am J Physiol Heart Circ Physiol* 269: H1528–H1537, 1995.
- Bazzoni G, Martinez Estrada O, and Dejana E. Molecular structure and functional role of vascular tight junctions. *Trends Cardiovasc Med* 9: 147–152, 1999.
- Bocci V. Efficient labeling of serum protein with <sup>131</sup>I using chloramine T. *Int J Appl Radiat Isot* 15: 449–456, 1964.
- Breviario F, Caveda L, Corada M, Martin-Padura I, Navarro P, Golay J, Introna M, Gulino D, Lampugnani MG, and Dejana E. Functional properties of human vascular endothelial cadherin (7B4/cadherin-5), an endothelium-specific cadherin. *Arterioscler Thromb Vasc Biol* 15: 1229–1239, 1995.
- Corada M, Mariotti M, Thurston G, Smith K, Kunkel R, Brockhaus M, Lampugnani MG, Martin-Padura I, Stoppacciaro A, Ruco L, McDonald DM, Ward PA, and Dejana E. Vascular endothelial-cadherin is an important determinant of microvascular integrity in vivo. *Proc Natl Acad Sci USA* 96: 9815–9820, 1999.
- Dejana E, Bazzoni G, and Lampugnani MG. Vascular endothelial (VE)-cadherin: only an intercellular glue? *Exp Cell Res* 252: 13–19, 1999.
- Dejana E, Corada M, and Lampugnani MG. Endothelial cell-to-cell junctions. *FASEB J* 9: 910–918, 1995.
- Dejana E, Valiron O, Navarro P, and Lampugnani MG. Intercellular junctions in the endothelium and the control of vascular permeability. *Ann NY Acad Sci* 811: 36–43, 1997.
- Dejana E, Zanetti A, and Del Maschio A. Adhesive proteins at endothelial cell-to-cell junctions and leukocyte extravasation. *Haemostasis* 26, Suppl 4: 210–219, 1996.
- Ellis CA, Tiruppathi C, Sandoval R, Niles WD, and Malik AB. Time course of recovery of endothelial cell surface thrombin receptor (PAR-1) expression. *Am J Physiol Cell Physiol* 276: C38–C45, 1999.
- Garcia JG, Davis HW, and Patterson CE. Regulation of endothelial cell gap formation and barrier dysfunction: role of myosin light chain phosphorylation. *J Cell Physiol* 163: 510–522, 1995.
- Grunwald GB. The structural and functional analysis of cadherin calcium-dependent cell adhesion molecules. *Curr Opin Cell Biol* 5: 797–805, 1993.
- Hovig T, Nicolaysen A, and Nicolaysen G. Ultrastructural studies of the alveolar-capillary barrier in isolated plasma-perfused rabbit lungs. Effects of EDTA and of increased capillary pressure. *Acta Physiol Scand* 82: 417–432, 1971.
- Kern DF, Levitt D, and Wangenstein D. Endothelial albumin permeability measured with a new technique in perfused rabbit lung. *Am J Physiol Heart Circ Physiol* 245: H229–H236, 1983.
- Kern DF and Malik AB. Microvascular albumin permeability in isolated perfused lung: effects of EDTA. *J Appl Physiol* 58: 372–375, 1985.
- Lampugnani MG, Corada M, Caveda L, Breviario F, Ayalon O, Geiger B, and Dejana E. The molecular organization of endothelial cell to cell junctions: differential association of plakoglobin, beta-catenin, and alpha-catenin with vascular endothelial cadherin (VE-cadherin). *J Cell Biol* 129: 203–217, 1995.
- Lampugnani MG and Dejana E. Interendothelial junctions: structure, signalling and functional roles. *Curr Opin Cell Biol* 9: 674–682, 1997.
- Lampugnani MG, Resnati M, Raiteri M, Pigott R, Pisacane A, Houen G, Ruco LP, and Dejana E. A novel endothelial-specific membrane protein is a marker of cell-cell contacts. *J Cell Biol* 118: 1511–1522, 1992.
- Lum H and Malik AB. Regulation of vascular endothelial barrier function. *Am J Physiol Lung Cell Mol Physiol* 267: L223–L241, 1994.
- Lum H and Malik AB. Mechanisms of increased endothelial permeability. *Can J Physiol Pharmacol* 74: 787–800, 1996.
- Malik AB and Lo SK. Vascular endothelial adhesion molecules and tissue inflammation. *Pharmacol Rev* 48: 213–229, 1996.
- Moy AB, Van Engelenhoven J, Bodmer J, Kamath J, Keese C, Gjaever I, Shasby S, and Shasby DM. Histamine and thrombin modulate endothelial focal adhesion through centripetal and centrifugal forces. *J Clin Invest* 97: 1020–1027, 1996.
- Niles WD and Malik AB. Endocytosis and exocytosis events regulate vesicle traffic in endothelial cells. *J Membr Biol* 167: 85–101, 1999.
- Rubin LL. Endothelial cells: adhesion and tight junctions. *Curr Opin Cell Biol* 4: 830–833, 1992.
- Schnittler HJ, Puschel B, and Drenckhahn D. Role of cadherins and plakoglobin in interendothelial adhesion under resting conditions and shear stress. *Am J Physiol Heart Circ Physiol* 273: H2396–H2405, 1997.
- Shasby DM and Shasby SS. Effects of calcium on transendothelial albumin transfer and electrical resistance. *J Appl Physiol* 60: 71–79, 1986.
- Srinivasan HB, Vogel SM, Vidyasagar D, and Malik AB. Protective effect of lung inflation in reperfusion-induced lung microvascular injury. *Am J Physiol Heart Circ Physiol* 278: H951–H957, 2000.
- Takeichi M. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* 251: 1451–1455, 1991.
- Telo P, Lostaglio S, and Dejana E. Structure of intercellular junctions in the endothelium. *Therapie* 52: 395–398, 1997.
- Tiruppathi C, Malik AB, Del Vecchio PJ, Keese CR, and Gjaever I. Electrical method for detection of endothelial cell shape change in real time: assessment of endothelial barrier function. *Proc Natl Acad Sci USA* 89: 7919–7923, 1992.
- Van Hinsbergh WM. Endothelial permeability for macromolecules. Mechanistic aspects of pathophysiological modulation. *Arterioscler Thromb Vasc Biol* 17: 1018–1023, 1997.
- Van Nieuw Amerongen GP, Draijer R, Vermeer MA, and van Hinsbergh VW. Transient and prolonged increase in endothelial permeability induced by histamine and thrombin: role of protein kinases, calcium, and RhoA. *Circ Res* 83: 1115–1123, 1998.
- Volberg T, Geiger B, Kartenbeck J, and Franke WW. Changes in membrane-microfilament interaction in intercellular adherens junctions upon removal of extracellular Ca<sup>2+</sup> ions. *J Cell Biol* 102: 1832–1842, 1986.