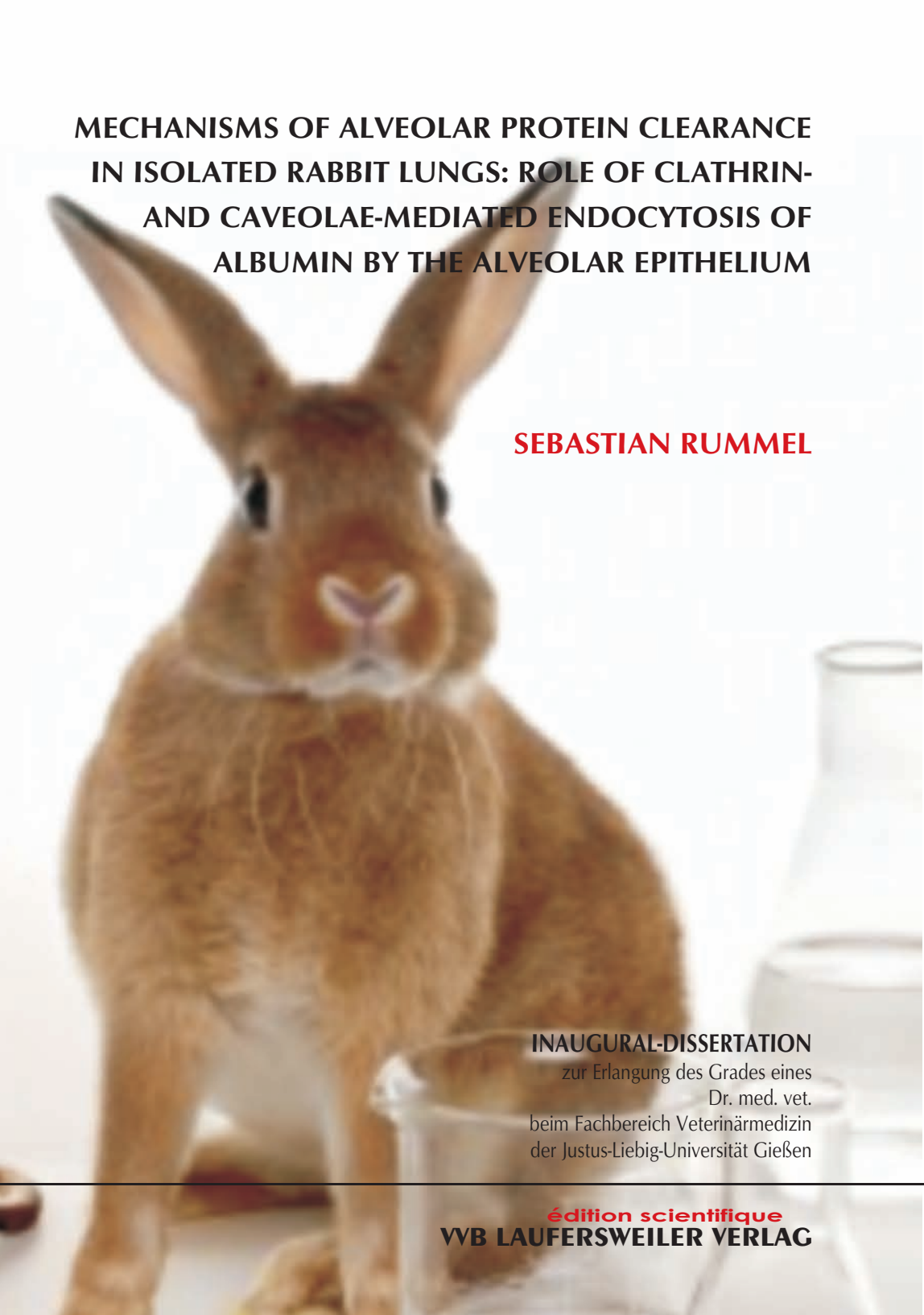
MECHANISMS OF ALVEOLAR PROTEIN CLEARANCE IN ISOLATED RABBIT LUNGS: ROLE OF CLATHRIN- AND CAVEOLAE-MEDIATED ENDOCYTOSIS OF ALBUMIN BY THE ALVEOLAR EPITHELIUM

SEBASTIAN RUMMEL



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Mechanisms of alveolar protein clearance in isolated rabbit lungs: Role of clathrin- and caveolae-mediated endocytosis of albumin by the alveolar epithelium

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eingereicht von

Sebastian Rummel

Tierarzt aus Wolgast

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Mit Genehmigung des Fachbereiches Veterinärmedizin
der Justus-Liebig-Universität Giessen

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Abbreviations

^{125}I-albumin	^{125}Iodined bovine serum albumin
A	asymptote of the exponential curve
A549	human lung adeno-carcinoma cell line
AAC	area above the curve
AEBSF	4-(2-aminoethyl)-benzenesulfonyl fluoride
AECC	American-European Consensus Conference
ALI	acute lung injury
ANOVA	one-way analysis of variances
AP180	assembly protein 180
AP-2	adaptor protein-2
ARDS	acute respiratory distress syndrome
ATPase	adenosine triphosphatase
AUC	area under the curve
BAL	broncho-alveolar lavage
BSA	bovine serum albumin
CFTR	cystic fibrosis transmembrane conductance regulator
Chlorprom	chlorpromazine
DMEM/F12	Dulbecco's modified Eagle's medium/Ham's F12 medium
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
DPBS-G	Dulbecco's phosphate buffered saline supplemented with glucose
e	exponential function of the curve
EDTA	ethylenediaminetetraacetic acid
EEA-1	early endosome associated protein-1
ELF	epithelial lining fluid
ENaC	epithelial sodium channel
Fc	fragment, crystallisable region
FCS	fetal calf serum

FiO_2	fraction of inspired oxygen
FITC	fluorescein isothiocyanate
gp60	albondin, albumin binding protein
GTPase	guanosine triphosphatase
HDL	high-density lipoprotein
I	iodine
IgA	immunoglobulin A
IgG	immunoglobulin G
intra vasc.	intra vascular
intra alv.	intra alveolar
LAP	left atrial pressure
LVP	left ventricular pressure
MDCK	Madine Darby canine kidney
min	minutes
MMAD	mass median aerodynamic diameter
Na,K-ATPase	Na,K-adenosine triphosphatase
neb	nebulization
NEM	*N*-ethylmalaimide
non spec	non specific
NSF	NEM-sensitive factor
PaO_2	partial arterial oxygen pressure
PAP	pulmonary artery pressure
PBS	phosphate buffered saline
PEEP	positive end-expiratory pressure
Phen ox	phenylarsine oxide
PO	phalloidin oleate
RLE-6TN	rat alveolar epithelial cells
rpm	revolutions per minute
SEM	standard error of the mean
SNARES	NEM-sensitive factor attachment protein receptor
VP	ventilation pressure

1. Introduction

1.1 The acute respiratory distress syndrome

The acute respiratory distress syndrome (ARDS) has an incidence of up to 75 per 100,000 individuals; in the United States of America, more than 150,000 cases are registered annually, and ARDS displays mortality rates of above 30 % (Zilberberg and Epstein 1998; ARDSNet 2000). Typical characteristics of ARDS include increased endothelial permeability (Kollef and Schuster 1995) and impaired epithelial barrier function (Matthay and Wiener-Kronish 1990); (Sznajder 1999), resulting in the accumulation of excess interstitial and/or alveolar edema fluid (**Figure 1**). Impaired edema fluid reabsorption is also associated with ARDS, and is essential for the resolution of this disease (Ware and Matthay 2001), since clearance of edema fluid is essential in patients with ARDS to survive (Matthay 2002); (Sznajder 2001). The underlying causes of ARDS include sepsis, pneumonia, trauma, multiple transfusions or acute pancreatitis. To set standard definitions, an American-European Consensus Conference (AECC) was convened in 1992. In their report, members of the conference defined acute lung injury (ALI) as the acute onset of arterial hypoxemia (partial arterial oxygen pressure [PaO_2]/fraction of inspired oxygen [FiO_2] ratio, under 300), a pulmonary artery wedge pressure less than 18 mmHg or no clinical evidence of left atrial hypertension, and bilateral infiltrates consistent with pulmonary edema on frontal chest radiography. The authors specifically noted that the pulmonary infiltrates could be mild. Acute respiratory distress syndrome is defined by the same criteria as ALI, but with more severe hypoxemia (PaO_2/FiO_2, lower than 200).

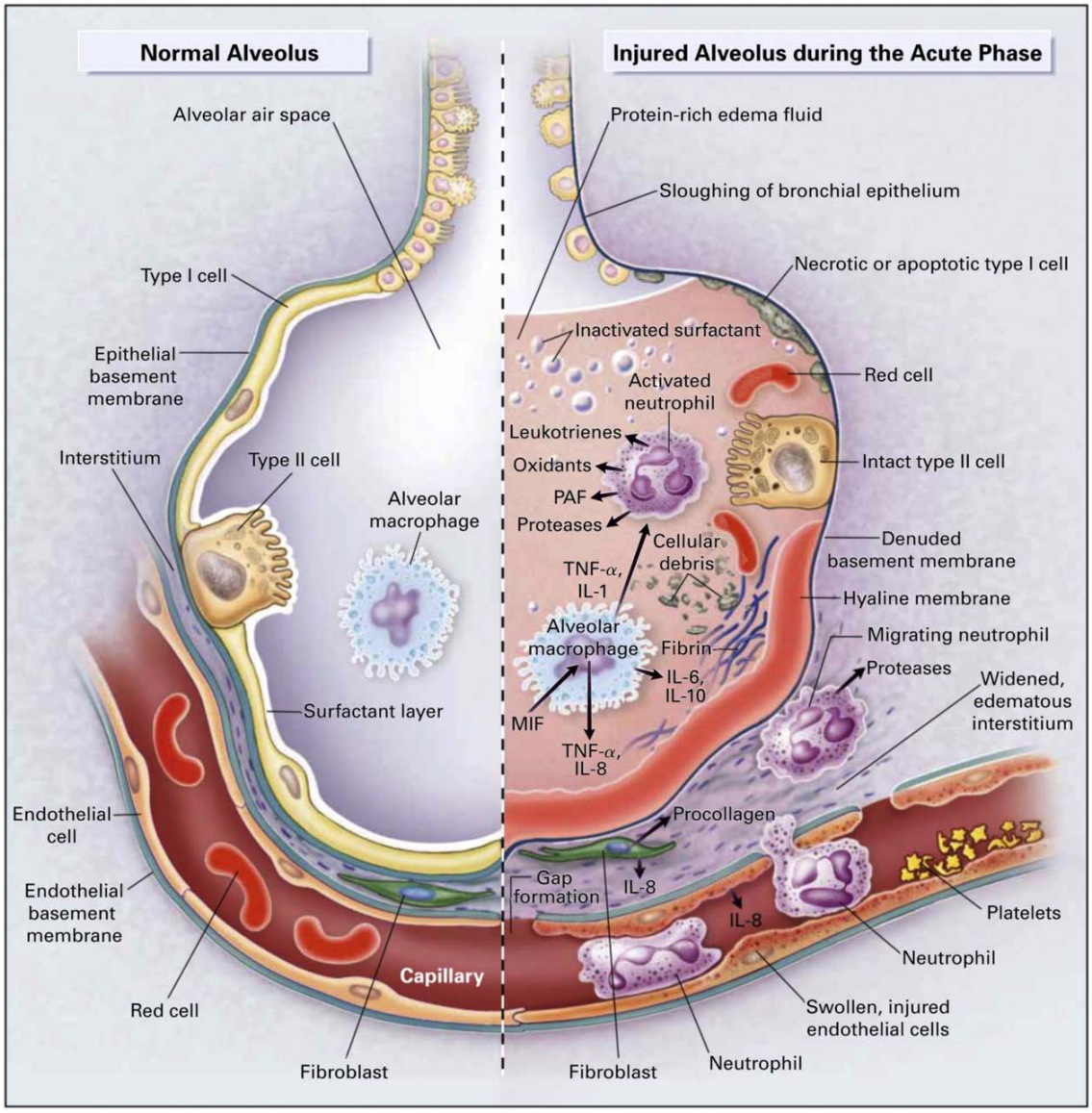

Figure 1. *Normal alveolar structure and function in comparison to circumstances in the acute phase of ALI/ARDS.*
On the left-hand side of the figure, the structure of the mammalian alveolo-capillary barrier in health is illustrated. The right-hand side of the figure illustrates inflammation and protein-rich alveolar edema formation in the alveolar space during the acute phase of ALI/ARDS. A depiction of cellular responses, mediators, as well as multiple molecular factors leading to imbalances between reparative and injurious pathways is illustrated. [Courtesy of Drs Ware and Matthay, reference (Ware and Matthay 2000)].

1.2 Alveolar fluid balance

In healthy humans, the apical side of the alveolar monolayer is covered by a thin fluid layer, termed the epithelial lining fluid (ELF). This fluid provides optimal surface tension regulation, and maintains basic host defense of the lung without affecting diffusion of gases. Thus, accumulation of fluid in the alveolar space severely impairs gas exchange. In ARDS, the excess alveolar liquid also includes massively increased amount of protein. Junctions of the epithelial monolayer become more permeable, followed by a considerable increase in the leakage of plasma proteins into the alveolar space. In healthy subjects the protein concentration in the ELF is approximately 8 – 10 % of the plasma concentration (Kim and Malik 2003). During edema formation in ARDS the plasma fractions in the ELF comprise protein levels between 40 - 90% (Hastings, Folkesson et al. 2004) of the plasma level (40-60 g/L). In contrast, edema fluid from patients with hydrostatic edema (transudate) contains less plasma protein compared to edema fluid from patients with permeability edema (exsudate), as assessed by a ratio of protein concentrations of edema fluid versus plasma (< 65% and > 65%, respectively) (Fein, Grossman et al. 1979). Protein concentration in the distal air spaces increases further during the recovery phase from alveolar edema because salt and water are cleared much faster than albumin (Matthay, Berthiaume et al. 1985; Matthay, Folkesson et al. 2002).

The primary mechanism driving fluid reabsorption from the alveolar space is the active transport of sodium from the airspace into the lung interstitium and pulmonary circulation (Matthay, Folkesson et al. 2002). Sodium uptake by alveolar epithelial cells occurs on the apical surface of the epithelium, primarily through amiloride-sensitive and -insensitive Na^+ channels (Matalon and O'Brodovich 1999; Kellenberger and Schild 2002), with subsequent transport out of the cell by Na^+,K^+-adenosine triphosphatease (Na^+,K^+-ATPase) located on the basolateral surface (Skou 1998; Sznajder, Factor et al. 2002). This active vectorial sodium flux generates an osmotic gradient, which leads to the movement of water from the alveolar space into the interstitium (Matthay,

Folkesson et al. 2002) and subsequently to the blood. Extensive research on human lungs (Sakuma, Okaniwa et al. 1994), in animal models (Serikov, Grady et al. 1993) and in both fluid-filled (Rutschman, Olivera et al. 1993) and ventilated isolated lung models (Vadasz, Morty et al. 2005) has demonstrated that alveolar fluid clearance is largely abrogated by hypothermia, probably by inhibition of active solute transport processes, and is inhibited by both amiloride, a specific inhibitor of some epithelial Na^+ channels (ENaC), as well as the Na^+,K^+-ATPase inhibitor ouabain (Ghofrani, Kohstall et al. 2001; Matthay, Folkesson et al. 2002). Furthermore, it has been well established (Saldias, Comellas et al. 1999; Azzam, Dumasius et al. 2002) that upregulation of ENaC and Na^+,K^+-ATPase increases active transepithelial Na^+ reabsorption, and thus the ability of the lungs to clear edema fluid (Therien and Blostein 2000; Kaplan 2002). Thus, it has been conclusively demonstrated that vectorial transport of sodium, mediated by apical sodium channels and the basolateral Na^+,K^+-ATPase, is the main contributor to alveolar liquid reabsorption

1.3 Effects of excess protein on alveolar epithelial function

Mechanisms by which excess protein is cleared from the alveolar space are not fully understood, although it has been documented that the velocity of albumin transport is significantly slower than that of sodium transport. As the concentration of protein further increases due to the differences in the transport rates of sodium and proteins, the clearence of edema fluid is hampered by the increased oncotic pressure generated by the excess protein in the alveolar space, thereby impeding recovery (Hastings, Folkesson et al. 2004). This alveolar flooding massively restricts alveolar gas exchange, resulting in alveolar hypoxia and systemic hypoxemia which have additional deleterious effects on the alveolar epithelial function and further impairs fluid balance of the lung (Vadasz and Sznajder 2006). Patients with ARDS exhibit elevated levels of precipitated protein in their distal air spaces. In alveolar edema fluid from non-survivors of

ARDS, the concentration of the precipitated protein was three times higher than in survivors (Bachofen and Weibel 1977). It is believed that the excess protein in the distal air spaces may play a role in hyaline membrane formation and thereby the development of end-stage fibrosis (Kobashi and Manabe 1993), (Tomashefski 1990). Possible modifications of excess proteins and degradation products like cationic amino acids and peptides may also have deleterious effects on the alveolar epithelium or transepithelial pathways and result in further edema formation (Kim and Malik 2003). Thus, the inability to remove excess protein from the alveolar space may play a major role in the poor outcome of patients with ARDS.

1.4 The anatomy of the lung

The alveolus and surrounding microcirculation are the primary sites of protein transport in the lung. Under physiological conditions, this area is composed of an epithelial and endothelial monolayer, which is separated by a minute interstitial space. The alveolar epithelium consist of type I and type II pneumocytes, and while these cell types are present in the lung in similar numbers, the type I cells represent ~95 % of the alveolar surface area (Mutlu and Sznajder 2005). These epithelial cells, together with their tight junctions, represent the primary barrier for solutes and electrolytes; compared with the endothelial monolayer, which is more permeable (Taylor and Gaar 1970).

1.5 Mechanisms of protein clearance from the distal airways

Several processes have been implicated in the mechanism that clears excess protein from the alveolar space. These include mucociliary clearance, endocytosis by alveolar macrophages, alveolar degradation with absorption of small molecular weight fragments by pneumocytes, and absorption across the alveolar and bronchial epithelium (Folkesson, Matthay et al. 1996). A schematic depiction of possible albumin clearance pathways is illustrated in **Figure 2**.

Mucociliary protein clearance with or without metabolic breakdown could clear proteins from the respiratory tract by moving proteins up the bronchial tree. Excess protein could reach ciliated epithelial cells in the terminal bronchioles via currents in the ELF (Kilburn 1968), however, a long half-time for mucociliary clearance (several days) from the alveolar compartment probably does not account for protein movement out of the alveolar space, since the latter process was found to be several orders of magnitude faster (Berthiaume, Albertine et al. 1989). Nevertheless, there is evidence for clearance via the mucociliary escalator, although with minimal - almost negligible - contribution to alveolar protein clearance, as assessed by detection of radio-labeled albumin or fibrinogen in the upper airways after intratracheal instillation (Meyer, Ottaviano et al. 1977; Meyer, Ottaviano et al. 1978).

Alveolar macrophages exhibit a larger endocytic capacity than do type II alveolar epithelial cells (Hastings, Folkesson et al. 1995) and their ability to degrade exogenous proteins and peptides (Steinman, Brodie et al. 1976) suggests that they may play a role in protein clearance from the alveolar compartment. However, clearance of proteins from the alveolar space is probably not mediated by alveolar macrophages, since in experiments where sheep lungs were instilled with autologous serum, thereby resulting in higher macrophage number in the air spaces, protein clearance remained unchanged (Matthay, Berthiaume et al. 1985). In that study, contribution of macrophages to protein clearance remained minor,

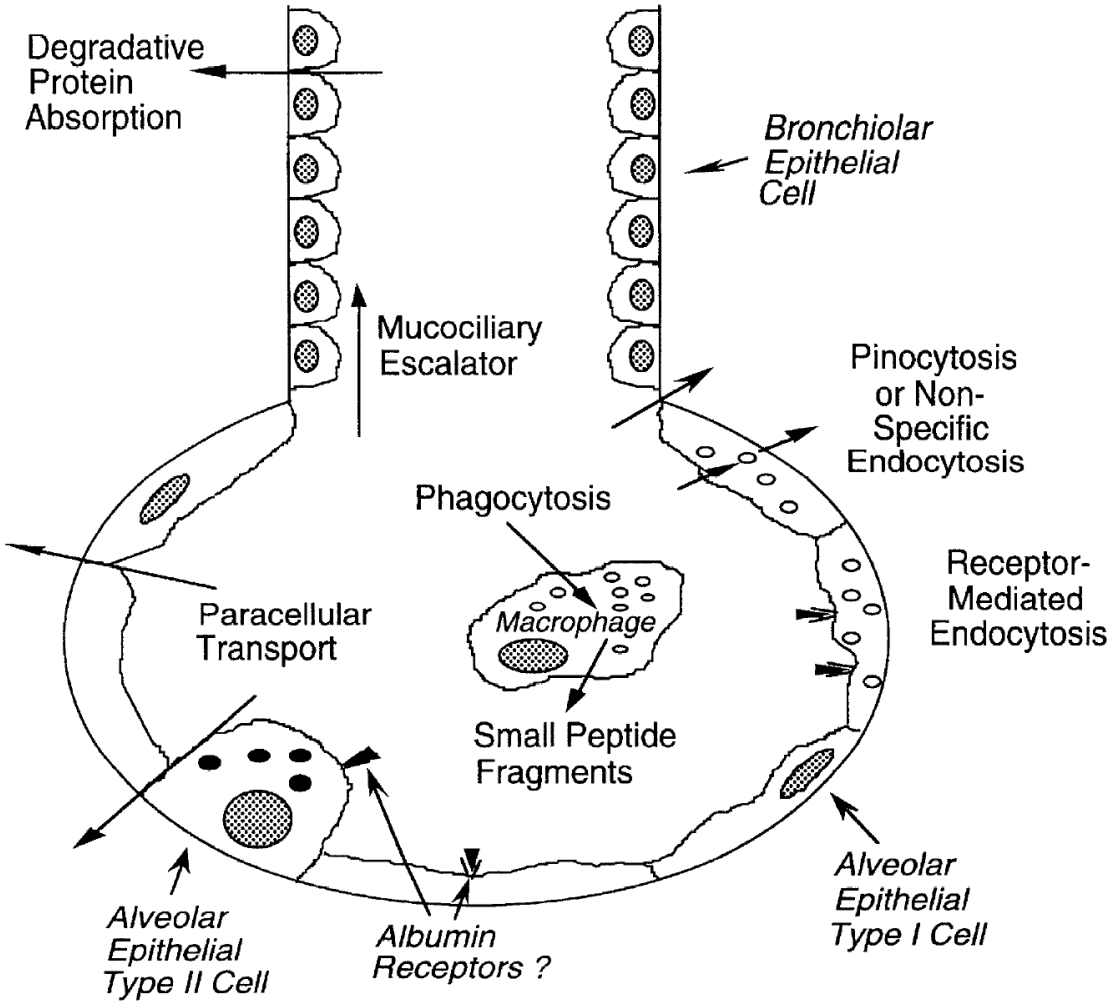

Figure 2. *Schematic depiction of possible albumin trafficking pathways accross the alveolo-capillary barrier*
Multiple possible mechanisms of albumin clearance from the alveolar compartment are depicted. Paracellular movement of protein either to the vasculature or to the lymphatic system are suggested, or elimination of albumin by macrophages. Furthermore, movement of albumin from the distal air spaces through alveolar epithelial cells is shown suggesting an active uptake of the molecule. [Courtesy of Dr Folkesson and collegues, reference (Folkesson and Matthay 1996)].

(1 - 2 days) but interestingly, in longer studies (2 - 6 days) the influx of macrophages into the alveolar compartment increased substantially, and was associated with increased quantities of protein tracer accumulation in

macrophages (Berthiaume, Albertine et al. 1989). However, despite of this increase macrophages appeared to play a minor role in alveolar protein clearance because the quantity of ^{125}I-albumin present in the phagocytic cells in the air spaces was less than 1 % of the instilled ^{125}I-albumin at all time points (Berthiaume, Albertine et al. 1989). In contrast, macrophages have been reported to play an important role in the clearance of surfactant apoproteins, and surfactant protein A (Ueda, Ikegami et al. 1995). Thus, alveolar macrophages may be important in the movement of some proteins from the alveolar compartment, in particular after long time periods, but their contribution in albumin removal from the distal air space is probably minimal.

Degradation of proteins in the ELF and absorption of small molecular weight fragments by pneumocytes could be also involved in protein clearance from the alveolar compartment. Thus, measurement of breakdown products in the alveolar space has been carried out by polyacrylamide agarose gel electrophoresis (Matthay, Berthiaume et al. 1985; Berthiaume, Albertine et al. 1989), trichloroacetic acid precipitation (Berthiaume, Broaddus et al. 1988; Berthiaume, Albertine et al. 1989), immunoprecipitation (Folkesson, Westrom et al. 1990; Folkesson, Westrom et al. 1993), and chromatography (Berthiaume, Albertine et al. 1989). Since the main classes of proteases in the alveolar space are metallo and serine proteases (Gross 1995; Greenlee, Werb et al. 2007), inhibition of these enzymes in the ELF is a powerful tool to examine albumin degradation in the alveoli. In particular, ethylenediaminetetraacetic acid (EDTA), a chelating agent, is widely used to sequester di- and trivalent metal ions. EDTA features four carboxylic acid and two amine groups which bind to metals, thereby inhibiting metallo proteases (Chiancone, Thulin et al. 1986), while 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), an irreversible inhibitor of serine proteases (Lunn and Sansone 1994), is often applied to block this protease class in the ELF. Additionally, peptidases are present in and on the apical membranes of pneumocytes (Nadel 1990; Ishii, Hashizume et al. 1991; Morimoto, Yamahara et al. 1993), thus, degradation might occurs through the action of proteases in the alveolus, in the cell membranes of the pneumocytes, or

after endocytosis. For example, there is evidence for degradation of the vasoactive intestinal polypeptide in the alveolus (Barrowcliffe, Morice et al. 1986). In contrast, several studies have suggested that bigger proteins (such as serum proteins including albumin) are probably transported intact across the alveolo-capillary barrier, as assessed by measurements of the integrity of instilled radio-labeled proteins in the perfusion (Berthiaume, Albertine et al. 1989; Folkesson, Westrom et al. 1990; Folkesson, Westrom et al. 1992). Alternatively, some investigators have suggested that protein transport across the excised canine bronchial epithelium to be accompanied by degradation (Johnson, Cheng et al. 1989) when proteins were nebulized. These contradictory findings highlight the need for further examinations in this field of investigation.

Under physiological conditions, the alveolo-capillary barrier is effectively impermeable to large solutes such as proteins, primarily due to the tight epithelial monolayer which exhibits a reflection coefficient for proteins of ~0.95, as opposed to the capillary endothelium, which is much more permeable, and has a reflection coefficient of ~0.7 (Gorin and Stewart 1979). In the alveoli, epithelial type I and type II cells are connected by a series of intercellular junctions. These apically-located intercellular junctions are referred to as the apical junctional complex which is composed of a tight junction and an adherens junction (Gumbiner 1987). Tight junctions are semipermeable gates regulating passive movement of luminal fluid and solutes through the paracellular pathway. This property of the tight junctions, in combination with transcellular vectorial transport, generates distinct environments in the opposing compartments. Thus, tight junctions are necessary to prevent dissipation of concentration gradients that exist between adjacent compartments (Laukoetter, Bruewer et al. 2006). Subjacent to tight junctions, adherens junctions, mainly composed of cadherins and catenins, are important in regulating intercellular adhesion (Mehta and Malik 2006). Thus, paracellular movement of plasma proteins through the alveolar epithelial monolayer is thought to be marginal under normal conditions.

1.6 Mechanisms of protein uptake by the alveolar epithelium

Recent studies have described an active albumin transport in various organs, including the proximal tubule cells of the kidney (Caruso-Neves, Kwon et al. 2005) and in the mammary gland of lactating mice (Monks and Neville 2004). Furthermore, it has been well documented that the bulk of albumin is taken up by the endothelial cells by endocytosis (Mehta and Malik 2006). In contrast, mechanisms of albumin uptake by the alveolar epithelium are poorly understood, although it is believed to occur by an endocytic process.

Endocytosis of macromolecules can be mediated by non-specific, fluid-phase uptake (macropinocytosis) where the degree of internalization is directly proportional to the concentration of the molecule present in the medium. During macropinocytosis, endocytic vesicles nonspecifically internalize small droplets of extracellular fluid. This process is extremely slow and cannot be saturated or competed out by the presence of other macromolecules (Conner and Schmid 2003). In the lung, horseradish peroxidase is taken up by alveolar epithelium by a fluid-phase transport, albeit at a much lower rate compared to albumin (John, Vogel et al. 2001; Kim and Malik 2003). In contrast, a much greater efficiency of endocytosis is achieved when dilute solutes are captured by specific high-affinity receptors (receptor-mediated endocytosis) (Conner and Schmid 2003). Up until now, two major receptor-mediated endocytic pathways have been described termed the caveolae-mediated and clathrin-dependent endocytosis.

1.7 Caveolae-mediated endocytosis

Caveolae are organelles with a diameter of 50 - 100 nm. In the endothelium, approximately 15 % of the total cell volume consists of caveolae, and up to 30,000 caveolae per cell exist (Johansson 1979). Although the basic structure of caveolae is independent of the cell type, their function and arrangement are cell type-dependent. Two subsets of caveolae have been described (Pelkmans and

Zerial 2005). Caveolae are either stored in stationary multi-caveolar structures at the plasma membrane, or undergo continuous cycles of fission and fusion with the plasma membrane. The process of endocytosis begins with the formation of caveolae. The integral membrane protein, caveolin-1, is required for the formation of caveolae (Drab, Verkade et al. 2001). Caveolin-2, a second participant, is also implicated in the caveolae cycle (Das, Lewis et al. 1999) although this hypothesis is controversial (Razani, Wang et al. 2002). In particular, some chemical reactions such as phosphorylation, palmitoylation, application of glycosphingolipids, and contact with cellular cholesterol may lead to caveolin-1 oligomerisation, regulation of intracellular localization, and sequestration to the plasma membrane (Sharma, Brown et al. 2004). Cholesterol is an important modulator of caveolae function, and conserves the structural integrity of the vesicle; thus filipin, a cholesterol binding agent, can impair the function of caveolae as this agent removes cholesterol from caveolae followed by its disassembly (Schnitzer, Oh et al. 1994).

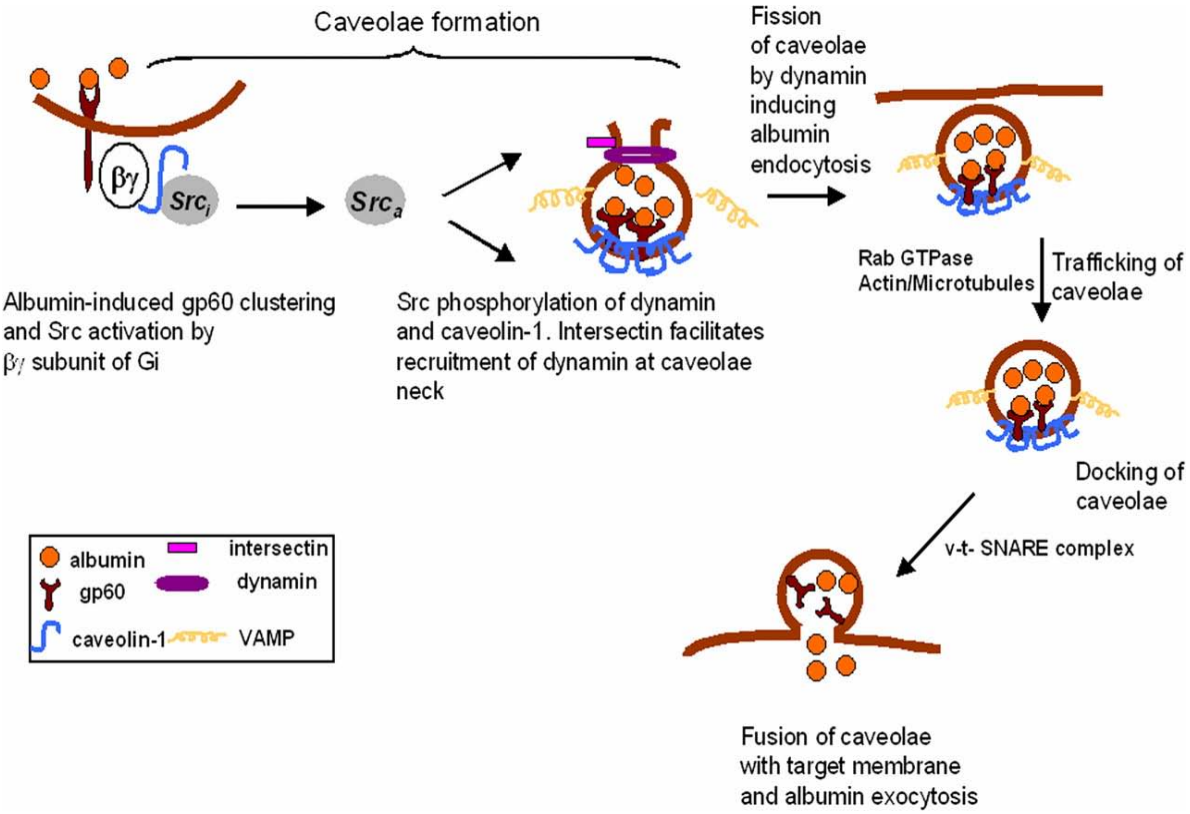

Figure 3. *Schematic depiction of the signaling mechanisms that regulate caveolae-mediated endocytosis, trafficking and exocytosis of albumin in endothelial cells.*
Albumin docking to the albumin binding protein (gp60) leads to activation of specific kinases. After phosphorylation of the molecular motor, dynamin, and caveolin-1, the phosphorylated molecules induce fission of caveolae from the membrane. The free vesicle moves through the cell and the SNARE machinery enables its fusion with the target membrane and thus exocytosis of albumin. [Courtesy of Drs Minshall and Tiruppathi, reference (Minshall, Tiruppathi et al. 2002)].

It has been conclusively demonstrated that caveolin-1 is a key molecule in the caveolae-mediated endocytosis of albumin in endothelial cells (Mehta and Malik 2006). The caveolae-mediated apical uptake of albumin is generally followed by trafficking of the protein to the basolateral side of the endothelium and its subsequent exocytosis, collectively referred as transcytosis (Mehta and Malik 2006). The soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) machinery may be required for caveolae-mediated transcytosis as a carrier system in the cells. The t-SNAREs are localized on the target (syntaxin and SNAP-25 family), whereas v-SNAREs (VAMP, vesicle-associated membrane protein) are components of the vesicle membrane. Contact between these two domains leads to fusion of the vesicle and target membranes, resulting in the release of albumin by exocytosis (Gerst 2003).

A key player in the regulation of exocytosis is the NEM-sensitive factor (NSF) (Whiteheart and Matveeva 2004). This adenosine triphosphatase (ATPase) initiates disassembly of v-SNARE and t-SNARE complexes. *N*-ethylmaleimide potently inhibits NSF by preventing the recycling activity of the ATPase, thereby inhibiting further exocytosis followed by a marked decrease of albumin transcytosis in cultured endothelial cells (Schnitzer, Allard et al. 1995) as well as *in situ* (Predescu, Horvat et al. 1994). However, several other molecules may play a role in the caveolae mediated endocytosis. For example Rab GTPases appear to be involved in membrane trafficking (Zerial and McBride 2001) and seem to be important regulators of all endocytic and exocytic processes. In addition actin and myosin as well as microtubules and molecular motors are part of the caveolae mediated endocytosis. A schematic depiction of the signaling mechanisms that regulate caveolae-mediated endocytosis, trafficking and exocytosis of albumin in endothelial cells is illustrated in **Figure 3**.

Several laboratories have described the existence of an albumin-binding protein (gp60 or albondin) on the surface of endothelial cells (Tiruppathi, Finnegan et al. 1996); (Tiruppathi, Song et al. 1997) and epithelial type II cells (Hastings, Folkesson et al. 2004). This gp60 receptor might be responsible for the promotion of endocytosis (Vogel, Minshall et al. 2001) and transcytosis of

albumin. It has been shown in endothelial cells that albumin docking to gp60 resulted in clustering of the receptor initiating uptake of the protein (Mehta and Malik 2006).

1.8 Clathrin-dependent endocytosis

Clathrin-dependent endocytosis has been described in several cells (Pearse 1976; Lin, Garbern et al. 1982) and is initiated by the clathrin coat assembly on the membrane followed by invagination and fission. Adaptor proteins, such as adaptor protein 2 (AP-2), epsin and amphiphysin, enable movement of vesicles away from the plasma membrane by facilitating fission (Wakeham, Ybe et al. 2000). Clathrin coats are key elements of the receptor-mediated (Conner and Schmid 2003) and fluid-phase endocytosis from the plasma membrane to the early endosomes. They are also involved in transport processes from the trans-Golgi network to the late endosome (Marsh and McMahon 1999). Phenylarsine oxide, a membrane-permeable phosphotyrosine phosphatase inhibitor, prevents formation of the clathrin-coated vesicle, thereby impairing clathrin-dependent endocytotic processes (Visser, Stevanovic et al. 2004). Chlorpromazine, another inhibitor of this pathway, impairs clathrin-dependent endocytosis by modulating AP-2 binding to the membranes leading to loss of coated pits from the surface of the cell (Wang, Rothberg et al. 1993). A schematic depiction of trafficking of macromolecules by clathrin-dependent pathways is illustrated in **Figure 4**.

Albumin binds to the gp60 receptor and this leads to a change in the conformation of the receptor. This modification enables the receptor to bind to AP-2 and to clathrin which is recruited by the assembly protein AP180 (Marsh and McMahon 1999), epsin and amphiphysin.

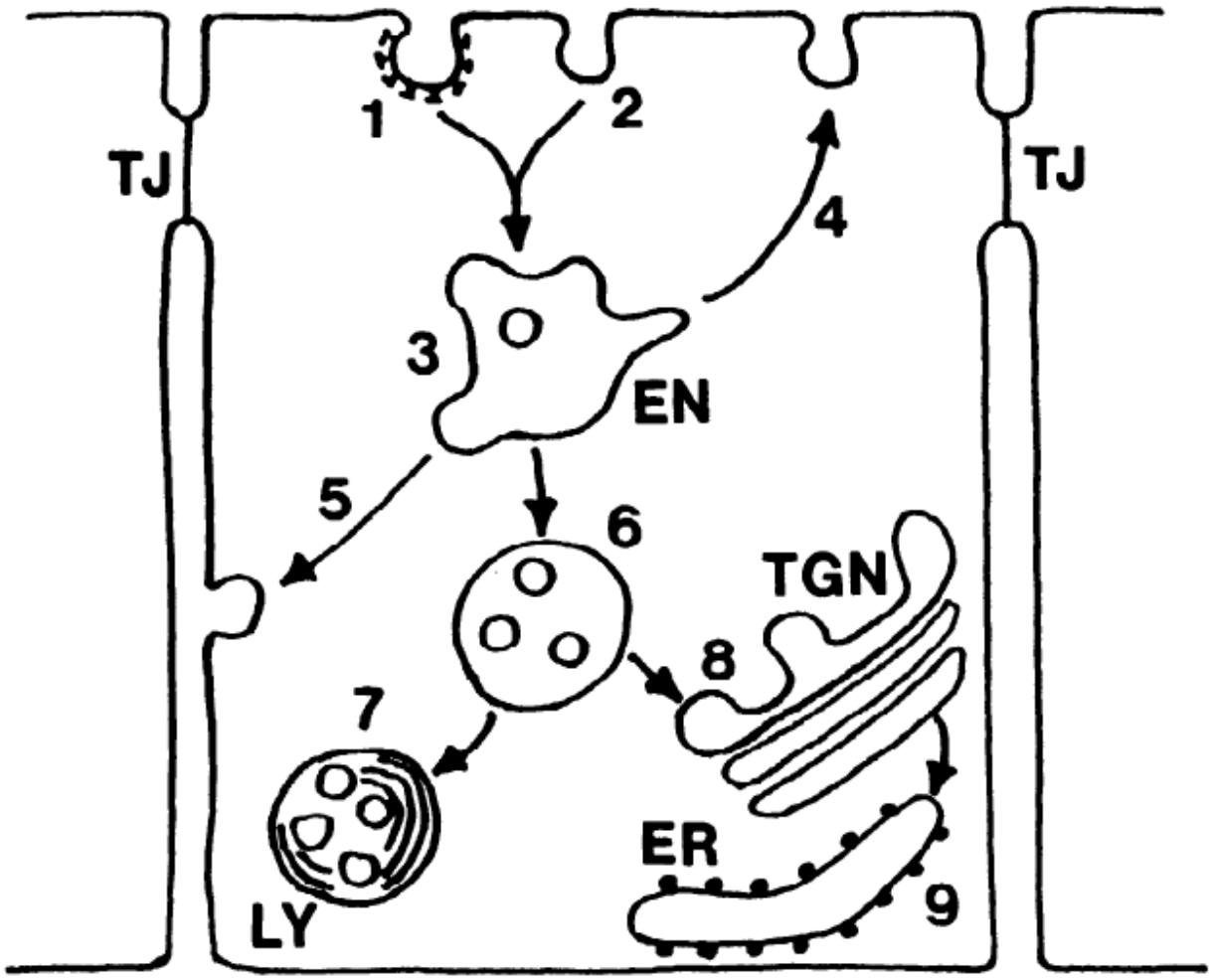

Figure 4. *Schematic depiction of trafficking of macromolecules by clathrin-dependent and caveolae-mediated pathways.*
Several possible pathways for albumin transport in polarized epithelial cells are illustrated in this figure. The initial step of albumin trafficking is clathrin-dependent (1) or caveolae-mediated (2) endocytosis of the molecule. This step is followed by vesicle fission and formation of the early endosome (3; EN). Content of the early endosome is either recycled to the internalized membrane domain and transported to the apical surface (4) or transporting the content without modification to the opposing surface (5; transcytosis). Alternatively a late endosome (6) is formed the content of which is either degraded by the lysosomes (7; LY) or further transported through the *trans*-Golgi-network (8; TGN) with final processing in the endoplasmatic reticulum (9; ER). [Courtesy of Drs Sandvig and van Deurs, reference (Sandvig and van Deurs 1996)].

While transport of albumin across the endothelium is primarily facilitated by caveolae-mediated endocytosis (Mehta and Malik 2006), protein uptake and its subsequent transport through the epithelial layer of the alveolo-capillary barrier is less understood, and remains controversial. This process is of particular interest since any excess protein accumulated in the alveolar space must first be transported through the epithelial monolayer before it can cross the endothelium. It was recently reported that transcytosis of albumin in epithelial cells is, at least in part, mediated by caveolae; since clearance of albumin from the alveolar space of isolated rat lungs was partially inhibited by the caveolae-disrupting agent filipin (John, Vogel et al. 2001). In contrast, mice in which the caveolin-1 gene was disrupted, and which do not have morphologically detectable caveolae, not only are viable, but they also do not show any defect in albumin transport (Drab, Verkade et al. 2001). However, it still needs to be addressed whether compensatory pathways exist that might have been induced in the absence of caveolin-1. Furthermore, a recent publication has suggested a role for clathrin in the uptake of albumin by cultured RLE-6TN rat alveolar epithelial cells (Yumoto, Nishikawa et al. 2006), which have several characteristics that are similar to alveolar type II cells (Driscoll, Carter et al. 1995). Thus, the exact mechanism by which alveolar epithelial cells endocytose proteins from the air spaces requires further elucidation.

1.9 Transcytosis of albumin through the alveolo-capillary barrier

Once proteins are taken up by the cell, they either cross the cell without alterations (transcytosis) (Mehta and Malik 2006) or undergo modifications, such as lysosmal or proteasomal degradation (Rivett 1990). Transcytosis is a strategy used by multicellular organisms to selectively move material between two different environments while maintaining the different compositions of distinct compartments in an actin-dependent manner (Tuma and Hubbard 2003). Thus, phalloidin oleate, a membrane-permeable peptide, that binds polymeric F-actin,

thereby stabilizing it and impairing the function of actin-rich structures, is a potent inhibitor of vesicle movement during endo- and trans-cytosis (Stenbeck and Horton 2004; Vadasz, Morty et al. 2005). In polarized cells, the net movement of molecules can be in either direction, apical to basolateral or the reverse, depending on the cargo and particular cellular context of the process. The best-studied example of transcytosis in the epithelium is the clathrin-dependent movement of the secretory immunoglobulin, IgA and its receptor (IgA-R) from the basolateral to the apical surface of cells (Mostov and Deitcher 1986). In particular, Madine Darby canine kidney (MDCK) cells have been studied extensively in order to characterize the intracellular intermediates. At least three compartments comprise the basolateral-to-apical transcytotic pathway of IgA-R in MDCK cells: basolateral early endosomes, a "common" endosome, and an apical recycling endosome (Wang, Brown et al. 2000).

Monensin, an ionophore that disrupts the structure of the Golgi apparatus, thereby inhibiting vesicular transport in the cell, is a potent inhibitor of transcytosis (Sakagami, Byron et al. 2002). Furthermore, monensin modulates the pH in vesicles resulting in a stoppage of vesicle recycling to the cell membrane (Hastings, Wright et al. 1994). Transcytosis of albumin has been described in detail in the endothelium, where caveolae appear to be the essential vesicle carriers responsible for the transport of albumin (Mehta and Malik 2006). However, whether albumin trafficking through the epithelium is mediated by transcytosis or the protein is degraded in the cell remains to be elucidated.

The mechanism by which proteins are cleared from the distal air spaces is largely unknown. Whether albumin moves through the alveolo-capillary barrier passively or is actively taken up by the epithelium is of debate. Since removal of protein from the alveolar space is of high clinical importance in the resolution of ARDS, understanding the underlying mechanisms may ultimately lead to new therapeutic strategies which could not only enhance transport of excess protein to the vascular side of the alveolo-capillary barrier but also result in a favorable outcome in ARDS.

1.10 Aims of our studies

In our studies we aimed to answer the following questions:

- Is albumin transport across the alveolo-capillary barrier an active or a passive process?
    - Is intact albumin taken up by the alveolar epithelium or degraded in the alveolar space?
    - Is albumin transport across the alveolo-capillary barrier uni-directional?
- If the transport is active, what is the mechanism of the uptake by epithelial cells in intact lungs?
    - Is the transalveolar albumin transport receptor-mediated?
    - Is uptake of albumin mediated by caveolae- or clathrin-dependent endocytic processes?
- Once albumin is taken up by the epithelium, what is the fate of the protein?
    - Is albumin transported intact through the alveolo-capillary barrier or degraded?

2. Material and Methods

2.1 The isolated, ventilated and perfused rabbit lung model

Physiological experiments were performed in isolated, ventilated and perfused rabbit lungs, a well established and widely accepted model for pulmonary research, which has been extensively used by our group and others in the past two decades (Seeger, Walmrath et al. 1994). This physiologically relevant model allowed us to investigate alveolar epithelial barrier function in an intact, *ex vivo* organ. Artificial ventilation and perfusion with a blood-free synthetic perfusate allowed us to conduct experiments under stable conditions for several hours that directly examinate changes in alveolo-capillary barrier function in the absence of any input from the rest of the organism. A schematic depiction of the isolated, ventilated and perfused rabbit lung model is illustrated in **Figure 5**.

2.1.1 Preparation and isolation of the lung and integration into perfusate circuit

Lungs were isolated from healthy, adult male rabbits (New Zealand White, Bauer, Neuenstein-Lohe, Germany). Animals weighing 3.0 ± 0.5 kg were employed. The ear vein of the rabbits was cannulated and an initial bolus of anesthetics was administered containing approximately 0.5 - 0.7 ml of a mixture of xylazine (Rompun 20 mg/ml; Bayer, Leverkusen, Germany) and ketamine (Ketavet 100 mg/ml; Pfizer, Karlsruhe, Germany) in a ratio of 3:2. Additionally, 1000 IU/kg heparin (Sodium heparin-25,000-ratiopharm, Ratiopharm, Ulm, Germany) was applied intravenously for anticoagulation. A further 1 ml of the anesthetics was administered stepwise over three minutes to achieve deep anesthesia, although allowing still spontaneous breathing. Animals were placed

in a supine position and the legs were fixed. Approximately 8 - 10 ml lidocaine (Xylocain 2 %, 20 mg/ml; AstraZeneca, Wedel, Germany) was injected subcutaneously into the ventral center of the neck to achieve local anesthesia, and a median incision from chin to the cranial thorax was performed. Thereafter, the trachea was exposed by blunt dissection and partially transected, and a tracheal cannula with an inner diameter of 3 mm was inserted; throughout, the animals were artificially ventilated with room air using a Harvard cat/rabbit ventilator (Hugo Sachs Elektronik, March Hugstetten, Germany). Subsequently, a second median incision was made from the center of the neck to the upper abdomen and a mid-sternal thoracotomy was performed. The xyphoid process was clamped and elongated, the diaphragm was dissected and the ribs were spread. Parts of the parietal pleura, the thymus and the bulk of the pericardium were removed to expose the heart, and the apex of the heart was clamped. A loose thread loop was formed around the ascendant aorta as well as pulmonary trunk; a bolus of 2 ml ketamine/xylazine was administered intravenously and a small incision was made in the upper right ventricle. Through the opening a fluid-filled pulmonary artery catheter of an inner diameter of 3 mm was inserted into the pulmonary artery and fixed with a thread loop; immediately after which the left atrium was opened by removing the apex of the heart and the descendent aorta was ligated. Lungs were perfused through the pulmonary artery catheter with a blood-free buffer, initially at 4 °C at a perfusion rate of 15 ml/min. Thereafter, the lungs, trachea and heart are excised *en bloc* from the thorax. The mitral valves and the *chordae tendineae* were dissected and a second perfusion catheter with an internal diameter of 4 mm was introduced via the left ventricle into the left atrium. A tobacco pouch suture (Mersilene; Ethicon, St-Stevens-Woluwe, Belgium) was used to fix this second catheter at the apex of the left ventricle such that no leakage from the catheterization sites, or obstruction of the pulmonary circulation occurred. After perfusing at least 1 l of buffer through the lungs to remove any blood from the system, the perfusion circuit was closed for the recirculation, while perfusion rate was increased from 15 to 100 ml/h temperature was increased to 37 °C.

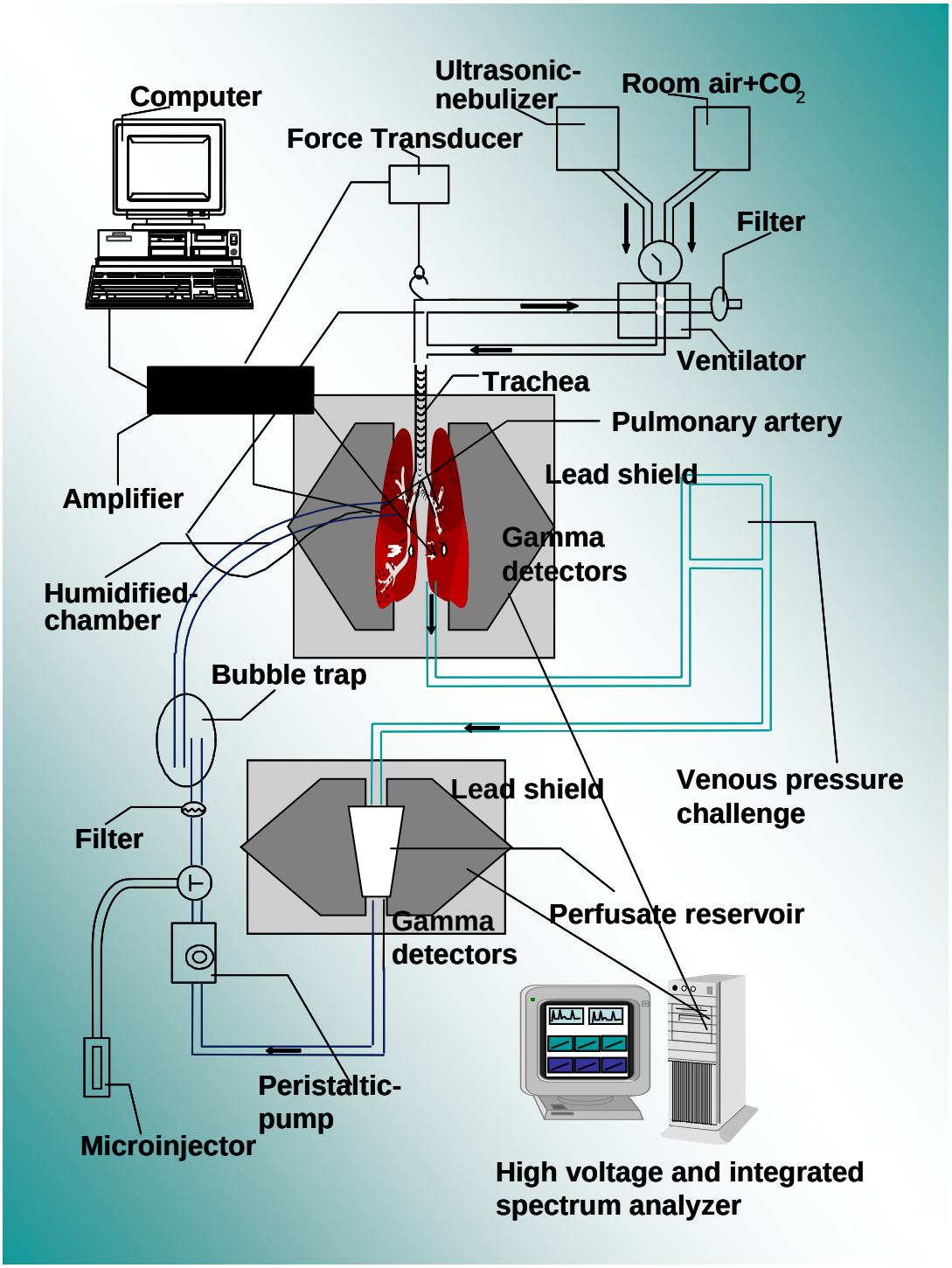

Figure 5. *Schematic depiction of the isolated, ventilated and perfused rabbit lung model* This scheme illustrates the isolated lung freely suspended from a force transducer in a heated chamber. Artificial respiration of the lung is depicted by the schematic assembly of the ventilation circuit including the ventilator, nebulizer and air reservoir. The arrows demonstrate the direction of the air flow. The perfusion of the organ is also shown in the figure, and arrows in the perfusion circuit point out the flow direction driven by a peristaltic pump. Lungs and the perfusate reservoir are surrounded by gamma detectors shielded by lead to allow detection only of radiation emitted from the lung and perfusate reservoir, respectively. Biophysical data are processed and amplified by a personal computer while the high voltage supply is delivered from a separate personal computer with an integrated power support.

2.1.2 Artificial respiration

During the lung preparation, natural respiration was replaced with artificial respiration with room air. Use of a Harvard cat/rabbit ventilator (Hugo Sachs Elektronik, March Hugstetten, Germany) enabled constant gas exchange under deep anesthesia. After lungs were isolated and separated from the rest of the organism, room-air was supplemented with 4.5 % CO_2 to maintain the pH of the recirculating buffer between 7.35 - 7.37. Ventilation parameters were set at 30 breaths/minute, a plateau pressure of 7.5 mmHg (0 mmHg was referenced at the hilum of the organ), a ratio between inspiration and expiration of 1:1 to maintain uniform ventilation. To prevent atelectasis, a positive end-expiratory pressure (PEEP) was set to 2 mmHg, resulting in a tidal volume of approximately 6 ml/body weight, which is considered protective (ARDSNet 2000).

2.1.3 The Perfusion of the isolate lung

A Krebs-Henseleit buffer (Elektrolytlösung IIN; Serag-Wiessner, Naila, Germany) containing 120 mM NaCl, 4.3 mM KCl, 1.1 mM Ka_2PO_4, 2.4 mM $CaCl_2$, 1.3 mM magnesium phosphate, 0.24 % [mass/vol] glucose, and 5 % [mass/vol] hydroxyethylamylopectin (for plasma expandation) was used for perfusion through a tubing system. Approximately 25 ml $NaHCO_3$ (Nabic 8.4 %, Braun, Melsungen, Germany) was added to maintain a pH of 7.35 - 7.37. Two independent reservoirs, allowing a perfusate change from one reservoir to the other without interrupting the circulation, were built into the system. Circulation of the perfusate was facilitated by a pump (Masterflex 7518-10; Cole Parmer, Vernon Hills, USA) at a flow rate of 100 ml/min. After passage through the pulmonary artery and perfusing the lungs, the perfusate left the lung through a catheter placed in the left atrium to the “venous“ part of the perfusion. Left atrial pressure (LAP) was adjusted by placing a catheter in the venous tubing system above the hilum. Temperature of the perfusate was maintained at 37 °C by using

a thermostat-controlled water bath (Thermo-Frigomix Braun, Melsungen, Germany) and a tube coil dipped into a 37 °C water bath. A pressure sensor was placed into the pulmonary artery to measure the pulmonary arterial pressure (PAP) and another pressure sensor into the left atrium to measure LAP. Immediately after the fluid-filled arterial catheter was placed into the pulmonary artery and throughout the rest of the preparation, lungs were perfused at a 15 ml/min flow rate and at a temperature of 4 °C. After the lungs and heart were isolated and removed *en block* from the thoracic cavity, the flow was increased step-wise to 100 ml/min and the temperature to 37 °C over approximately 10 minutes. During this time lungs were perfused with at least 1 l non-recirculating buffer to remove any residual blood cells from the circulation. A filter of 0.2 μm mesh size (Pall Cardioplegia; Terumo, Eschborn, Germany) was also placed into the circulation system to eliminate remaining cells or pyrogenic aggregates. After perfusion reached the desired 100 ml/min flow rate and the buffer was heated to 37 °C the filter system was removed and the perfusion buffer was recirculated. The volume of the recirculating buffer in the system was 300 ml. At the end of the preparation and after the lungs were freely suspended from a force transducer LAP was set at 2 mm Hg. Throughout the experiment, a bubble trap was present in the perfusion system to prevent any air embolisms in the circulation.

2.1.4 Nebulization

Substances were delivered to the alveolar space with an ultrasonic nebulizer (Optineb, NEBU-TEC, Elsenfeld, Germany). The nebulizer was connected directly to the inspiration loop of the ventilator. Filters (Iso-Gard Filter S, Hudson, Temecula, USA) were mounted in the outlet of the inspiration and expiration loop to prevent contamination of the environment. Characterization of the nebulizer revealed an average particle size of 3.2 μm as assessed by measurements of the mass median aerodynamic diameter (MMAD). During a

10 min aerosolization period, 1.6-1.8 ml of aerosol was generated, and a fraction (~60%) of this aerosol (~1 ml) reached the lung the bulk of which was deposited into the alveolar space (**Figure 6** (Vadasz, Morty et al. 2005; Vadasz, Morty et al. 2005).

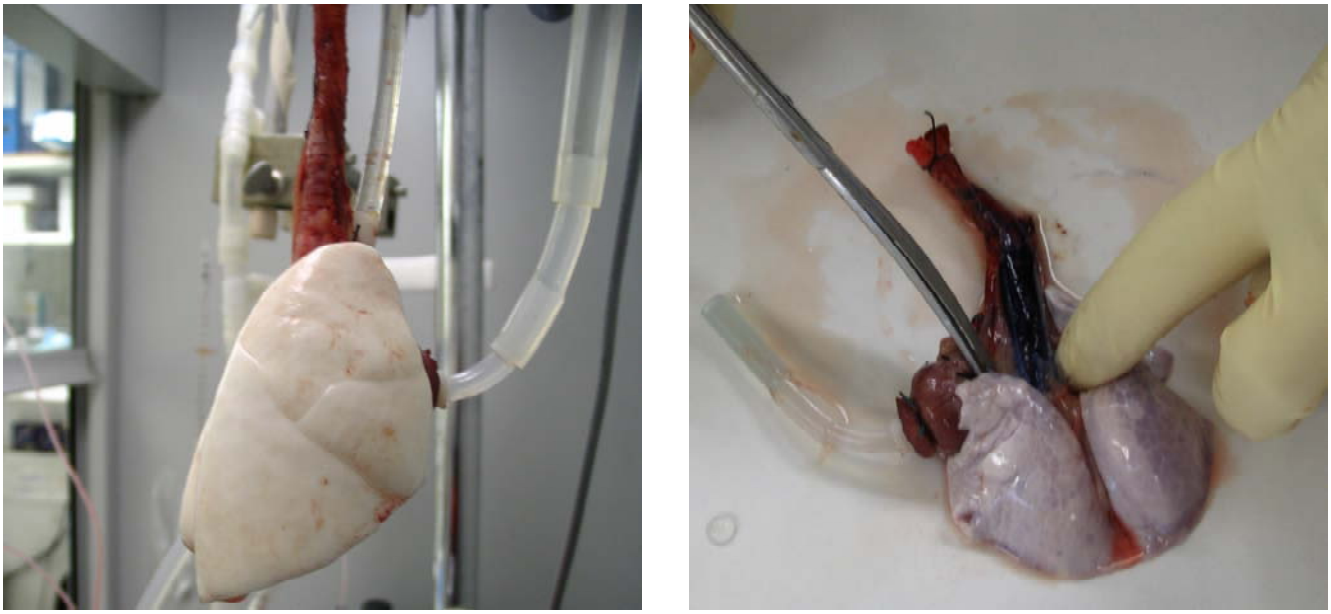

Figure 6. *Distribution of aerosolized Evans blue dye in the isolated, ventilated and perfused rabbit lung*
The picture on the left-hand side illustrates a control lung that has been nebulized with physiological saline. The right panel shows dissemination of aerosolized liquid containing Evans blue dye, which was used to evaluate the efficiency of ultrasonic nebulization, in the isolated lung. Uniform staining of the peripheral tissue was evident, suggesting that nebulization is a powerful tool to administer substances to the alveolar compartment.

2.1.4.1 Tracers

^{125}I-bovine serum albumin (^{125}I-albumin) (PerkinElmer, Boston, USA) was used to monitor protein transport in the lung, as well as in cell culture experiments. The specific activity of the ^{125}I-albumin was 3 μCi/μg protein. Approximately 6 μCi (and thus 2 μg) of albumin was deposited into the alveolar space during the 10-min nebulization. About 12 μCi of [^{3}H]mannitol (PerkinElmer, Boston, USA) was applied to the distal air spaces to measure paracellular transport of small solutes. In the case of isolated, ventilated and perfused rabbit lung experiments, tracers were deposited into the alveolar space by ultrasonic nebulization; while in cell culture experiments solute, radio-labeled substances were directly added to the cell culture medium.

2.1.4.2 Determination of the intactness of ^{125}I-albumin

Intactness of ^{125}I-albumin was assessed by two independent methods. Trichloroacetic acid (TCA) was applied to precipitate protein content of BAL and perfusate samples and samples of non-nebulized aliquots of ^{125}I-albumin, as described previously (Hastings, Folkesson et al. 1995). Briefly, 1 ml samples were precipitated with 2 ml 20 % TCA [vol/vol], and spun at 14,000 rpm for 10 min in a Hettich Micro 22 R centrifuge (Tuttlingen, Germany). Supernatants, which contained the cleaved fractions of ^{125}I-albumin, were transferred to a new Eppendorf tube and the amount of γ-emission was quantified by a γ-counter (Packard, Dreieich, Germany). The amount of γ-emission was compared to samples, which contained both the supernatant and the pellet and thus included both cleaved and intact fractions of the radio-labeled protein. The amount of intact ^{125}I-albumin in BAL and perfusate samples was also determined by centrifugal filters (Amicon; Centricon, Bedford, USA) with a molecular cut-off of 50 kDa, as recommended by the manufacturer. From each BAL and perfusate sample 2 ml was administered to the sample reservoir of the filter device and

than spun in a Rotina 46 R (Hettich, Kirchlengern, Germany) centrifuge at 4,000 rpm for 10 min. Since intact albumin is ~69 kDa, samples were separated into two fractions by centrifugation. The first fraction, containing all cleaved ^{125}I-albumin, appeared in the filtrate vial; and a second fraction, which contained intact ^{125}I-albumin, remained in the sample reservoir. The amount of γ-emission in these fractions was quantified by a γ-counter (Packard, Dreieich, Germany).

2.1.4.3 Measurement of Tracer Exchange

Gamma-detectors (Target System Electronic, Solingen, Germany) were used to detect ^{125}I-albumin and were connected to an automated high-voltage power supply integrated into a personal computer system with data processing. Four detectors, two of them placed around the lungs and two around the perfusate, registered movements of ^{125}I-albumin in real-time. The [^{3}H]mannitol tracer kinetics were monitored by perfusate sampling at 0, 5, 10, 20, 40, 80 and 120 min after the end of the nebulization step and was quantified by scintillation counting in a Canberra Packard β-counter (Packard, Dreieich, Germany).

2.1.4.4 Calculation of Tracer Kinetics

Counts of the gamma emitter ^{125}I-albumin were recorded continuously by gamma-detectors every 30 s over the entire time-course of the experiment. The starting point of each tracer clearance measurement in the lung, as well as in the perfusate, was set at the end of the nebulization, and at this time point the amount of radiation in the lung was referenced to 100 % and the amount of radiation in the perfusate to 0 %. The clearance rate from the lungs, and transit into the perfusate, were calculated by measuring the area above the curve (AAC) as determined by the following equation:

$$AAC = [(100-A) \times T]- \{[-(100-A) / e] \times [1-\exp (e \times T)]\}$$

where “A” is the asymptote of the exponential curve, “T” is the time; and “e” is the exponential function of the curve. A correction for additive radiation over the lung was applied (in vessels of the organ and tubes close to the lung; measured by lung detectors, approximately 15 ml). However, this additive activity did not exceed 5.5 % of the radioactivity deposited into the alveoli. Because different tracer yields were detected over the lung and the perfusate (for geometric reasons), detectors surrounding the reservoir were calibrated to match the yield of tracer detection over the lung, thereby allowing direct comparison of the obtained values for ^{125}I-albumin tracer quantities. Discontinuous sampling of the perfusate enabled assessment of intactness of the alveolo-capillary barrier, as measured by [^{3}H]mannitol movement into the circulation, a marker of passive paracellular permeability.

2.1.5 Bronchoalveolar lavage

At the conclusion of each experiment a bronchoalveolar lavage (BAL), was performed. Immediately after determination of ventilation and perfusion, the left lung was ligated and 30 ml of isomolar mannitol (Mannit-15%, Serag Wiessner, Naila, Germany) was instilled into the right lung and gently reaspirated three times to recover lavage fluid. Approximately 75 – 80 % of the instilled fluid was recovered, and the entire procedure took less than 30 s. The recovered fluid was then centrifuged at 1000 revolutions per minute (rpm) in a Hettich Micro 22 R centrifuge, (Tuttlingen, Germany) for 10 min to pellet any cellular debris from the lavage fluid.

2.1.6 Monitoring parameters in the isolated, ventilated and perfused lungs

Ventilation pressure (VP), left atrial pressure (LAP) and pulmonary artery pressure (PAP) were detected with catheters placed in the expiration loop of the ventilator, the left atrium and the pulmonary artery. An electromechanical pressure converter (Combitrans, Braun, Melsungen, Germany) allowed digitalization of these signals. During every experiment in the isolated lung model, the weight of the organ was measured and changes in the weight were detected by a force transducer. All analog signals were amplified, converted to digital signals and the use of the PlotIT 3.1 software (Scientific Programming Enterprises, SPE, USA) enabled an on-line graphical demonstration of LVP, PAP, VP and weight on a personal computer during the experiment **(Figure 7)**.

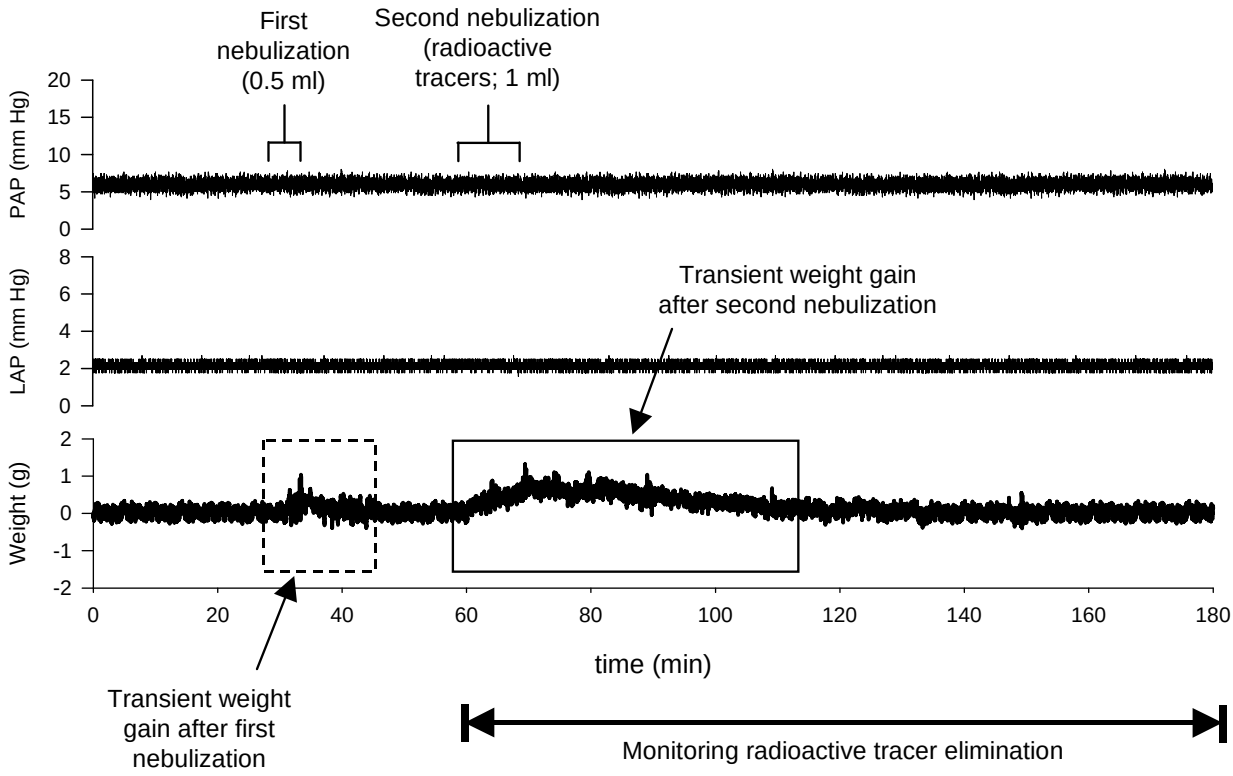

Figure 7. *Schematic depiction of the experimental protocol in the isolated, ventilated and perfused rabbit lungs.*
Original recordings of pulmonary artery pressure (PAP), left atrial pressure (LAP) and lung weight of a control experiment are illustrated. After a 30 min steady-state period, lungs were first nebulized with pharmacological agents or their vehicles. After an additional 30 min period lungs were aerosolized with physiological saline containing ^{125}I-albumin and [^{3}H]mannitol, and the clearance of the radio-labeled albumin was monitored for 120 min in real-time by γ-detectors placed around the lungs and the perfusate reservoir. Movement of [^{3}H]mannitol across the alveolo-capillary barrier was detected from perfusate samples at timed intervals. Both nebulizations resulted in a transient weight gain of the lung. The first aerosolization deposited ~0.5 ml liquid to the distal air space, hence the 0.5 g transient weight gain (dashed box). A second transient weight gain of the lung induced by the second nebulization (fluid challenge approximately 1 ml) is indicated by the solid box.

2.1.7 Substances applied in the physiological model

Pharmacological agents applied to the isolated, ventilated and perfused rabbit lung model are listed in Table I. All chemicals listed in Table I were from Sigma (St. Louis, USA).

Table I

Chemical agents applied to the isolated rabbit lungs

Application	**Agent**	**Vehicle**	**Final concentration**	**Administration**
Competitors	BSA	NaCl	2 mg/ml	ELF
	BSA	NaCl	20 mg/ml	Perfusate
Protease inhibitors	EDTA	NaCl	10 µg/ml	ELF
	AEBSF	DMSO	0.25 mg/ml	ELF
Caveolae inhibitors	NEM	NaCl	5 µg/ml	Perfusate
	Filipin	DMSO	2 µg/ml	Perfusate
Clathrin inhibitors	Chlorpromazine	NaCl	5 µg/ml	ELF
	Phen ox	DMSO	30 µg/ml	ELF
Transcytosis inhibitors	Phalloidin oleate	DMSO	1 µg/ml	ELF
	Monensin	Methanol	15 µg/ml	ELF

2.1.8 Experimental protocol for the isolated, ventilated and perfused lungs

The time-course of all experiments was 180 min. Experimental protocols are illustrated in **Figure 7**. Lungs included in our study had no signs of hemostasis, edema or atelectasis; exhibited a constant mean PAP and VP in the normal range; and were isogravimetric during an initial steady-state period of 30 min. After the conclusion of the steady-state period, lungs were nebulized with an Optineb ultrasonic nebulizer (Nebu-Tec, Elsenfeld, Germany), connected directly to the inspiration loop of the ventilator. Either pharmacological agents (diluted in dimethyl sulfoxide (DMSO) or in methanol in physiological saline (1 % [vol/vol] in both cases) or saline in the presence of the vehicles (for control experiments) were aerosolized into the lungs. A list of these pharmacological agents together with their suppliers and final concentrations in the ELF and perfusate are listed in **Table I**. The first nebulization (duration 5 min) deposited 0.5 ml of fluid into the alveolar space, with a concomitant 0.5 g transient increase in lung weight, attributable to this fluid that was nebulized into the lung (**Figure 7** *dashed box*). In some experiments this nebulization step was replaced by administration of pharmacological agents to the perfusate in order to target the vascular compartment of the lung.

Lungs were then allowed to re-establish baseline conditions for 30 min after which radioactive tracers were applied by a second ultrasonic nebulization over 10 min. This second nebulization deposited 1 ml of fluid into the alveolar space, with a concomitant 1 g increase in lung weight, attributable to this fluid that was nebulized into the lung (Figure 7, *solid box*). Transport of ^{125}I-albumin was monitored real-time from the beginning of the tracer nebulization until the conclusion of the experiment with the help of gamma-detectors, while movement of [^{3}H]mannitol was assessed by discontinuous perfusate sampling, as described in section *2.1.4.3.* Additionally, at the conclusion of the experiment, a BAL was performed. As described in section *2.1.4* experiments were conducted at 37 °C, however, selected experiments were performed at 22 °C or at 4 °C to assess temperature dependence.

In some additional experiments, fluorescein isothiocyanate (FITC)-labeled albumin influx from the perfusate into the alveolar space was measured to address whether movement of albumin was bi-directional. The FITC-albumin was administered to the perfusate (at a final concentration of 0.16 mg/ml) 30 min after the end of the steady-state period. The FITC-albumin concentrations were determined from BAL fluids (200 µl) in a Fusion microplate spectrofluorimeter (Packard; Dreieich, Germany) at an emission wavelength of 480 nm and an excitation wavelength of 520 nm, as described previously (Lecuona, Saldias et al. 1999).

2.2. Cellular experiments

Experiments in alveolar epithelial monolayers were performed to further dissect the mechanisms by which uptake of albumin by the alveolar epithelium occurred, and to verify the results that were obtained from the isolated, ventilated and perfused rabbit lung model. For these experiments, cultured human epithelial cells (A549; ATCC-No.: CCL-185) were employed. This cell-line of human adenocarcinoma origin has been extensively studied by several research groups and represents a both well-characterized and excellent model system for primary alveolar epithelial type II cells.

2.2.1 Culturing the cells

The A549 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F12; 1:1, GIBCO, Invitrogen, Karlsruhe, Germany) supplemented with 10 % [vol/vol] fetal calf serum (FCS; PAA Laboratories, Egelsbach, Germany), 1 % [vol/vol] glutamine, 1 % [vol/vol] penicillin/ streptomycin (both from PAN-Biotech, Aidenbach, Germany), 1 % [vol/vol] non-essential amino-acids and 1 % [vol/vol] vitamins (both from GIBCO,

Invitrogen, Karlsruhe, Germany). Cells were grown in 75 cm^2 tissue culture flasks (Cellstar; Greiner Bio-One, Frickenhausen, Germany) for two days to 80 - 90 % confluence (approximately 4 - 5 million cells) in a Haereus cell culture incubator (Haereus Instruments, Hanau, Germany) at a temperature of 37 °C, 80 - 95 % relative humidity and 5 % carbon dioxide. For subculturing, culture medium was removed and the cells were rinsed with DMEM/F12 medium twice, after which 5 ml of 0.25 % trypsin-EDTA (PAN-Biotech, Aidenbach, Germany) was added and cells were placed in a cell culture incubator for an additional 5 minutes to enhance cell detachment. To achieve complete cell detachment, cells were resuspended in medium containing 1.5 ml FCS and 5 ml DMEM/F12 in the flasks. The suspended cells were then transferred to a 15 ml conical tube (BD Biosciences, Heidelberg, Germany) and were centrifuged in a Rotina 46 R centrifuge (Hettich, Kirchlengern, Germany) at 1200 revolutions per minute at 15 °C for 10 min. The supernatant was removed and discarded and the cell pellet was resuspended in culture media and 1.5 million cells were seeded in 75 cm^2 tissue culture flasks for further subculturing. For our experiments, A549 cells of passage 1 to 10 were employed and approximately 125,000 cells were placed into each well of a six well tissue culture plate (Cellstar; Greiner Bio-One, Frickenhausen, Germany). Under these culture conditions, A549 cells doubled approximately every 22 h. Therefore, at the time of the experiments, 44 - 48 h after plating, approximately 500,000 cells per dish were counted.

2.2.2 Pharmacological agents applied to the cell culture model

Pharmacological agents applied to monolayers of A549 cultured epithelial cells are included in **Table II**. All chemicals listed in Table I and II were from Sigma (St. Louis, USA).

Table II

Chemical agents applied to A549 cells

Application	Agent	Vehicle	Final concentration
Competitor	BSA	PBS	20 mg/ml
Caveolae inhibitors	NEM	PBS	100 µg/ml
	Filipin	DMSO	2 µg/ml
Clathrin inhibitors	Chlorpromazine	PBS	30 µg/ml
	Phen ox	DMSO	5 µg/ml

2.2.3 Protocol of cellular experiments

At the beginning of the experiments, media was removed from the culture plates and cell layers were rinsed briefly with Dulbecco's phosphate buffered saline (DPBS; PAN Biotech, Aidenbach, Germany) supplemented with 0.1 mM $CaCl_2$ dihydrate (Calbiochem, San Diego, USA) 0.5 mM $MgCl_2$ $6H_2O$ and 5 mM glucose (both from Sigma, St. Louis, USA) (DPBS-G). Cells were then pre-incubated for 10 min with 2 ml of DPBS-G followed by a pre-incubation with pharmacological agents or their vehicles. The details of treatments with these agents are described in **Table II**. After treatment of the cells with these agents 2 ml of DPBS-G containing ^{125}I-albumin was applied to the cells in the presence or absence of these agents for different time periods. ^{125}I-albumin uptake was terminated after 2.5, 5, 10, 30 or 60 min by aspiration of the medium and the addition of ice-cold DPBS. Wells were washed thoroughly with ice-cold DPBS

to remove any residual tracer from the surface of the cells. Cells were then solubilized by treating them with a cell culture lysis reagent (Promega, Madison, WI, USA) at 4 °C for 5 minutes, and ^{125}I-albumin in the cell extracts was quantified by γ-emission counting in a Packard γ-counter (Packard, Dreieich, Germany).

2.2.4 Assessment of cell viability

Cell viability was tested with the Trypan Blue exclusion dye method (Perry, Epstein et al. 1997). Cells were plated in six-well plates exactly as described above and viability of control cells and cells exposed to drugs or their vehicles alone were assessed by adding 50 µl of Trypan Blue solution (0.4 % [mass/vol] Trypan blue in PBS) in culture medium. After 1 – 2 min, the number of dead cells, which retained the dye, was compared to the total number to calculate the mortality percentage.

2.3 Statistical analysis of data

Numerical values are given as the mean ± standard deviation (SD). Comparisons between two groups were made using an unpaired, two-tailed Student's *t-test*. Intergroup differences of three or more experimental groups were assessed by using a one-way analysis of variance (ANOVA) with *post hoc* Dunnett-test, when comparing values only to controls or Tukey-test for multiple comparisons. p values < 0.05 were considered significant. GraphPad Prism 4 for Windows software (GraphPad Software; San Diego, USA) was used for data plotting and statistical analysis.

3. Results

3.1 Clearance of albumin from the alveolar space of isolated rabbit lungs is an active process

At 37 °C, 29.8 ± 2.2% of the ^{125}I-albumin deposited to the alveolar space was cleared from the lungs by the end of the experiment (**Figure 8**). This movement of albumin was temperature-dependent, as experiments conducted at 22 °C and at 4 °C resulted in a significantly lower clearance of the nebulized ^{125}I-albumin (16.2 ± 1.1 % and 3.7 ± 0.4 %, respectively; **Figure 8**). Albumin clearance at 4 °C is attributable exclusively to passive transport processes, because active processes are shut down at this temperature (Rutschman, Olivera et al. 1993). Passive epithelial paracellular permeability for solutes, as assessed by [^{3}H]mannitol clearance from the lung, was not significantly affected by low temperature, when compared with control lungs at 37 °C (**Figure 9**). Thus, we reasoned that clearance of ^{125}I-albumin from the lungs at 37 °C that was not cleared at 4 °C was attributable to active processes, because passive processes were unaffected under all experimental conditions. Approximately 88 % of the ^{125}I-albumin transport was active, as measured by the difference between the area above the curve for the experiments conducted at 37 °C and 4 °C (**Figures 8 and 9**).

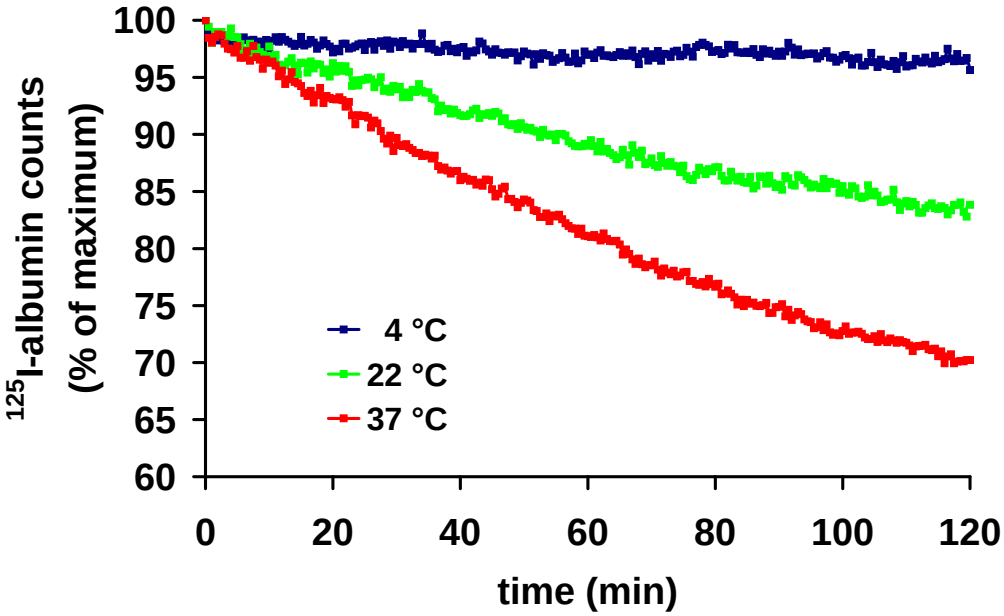

Figure 8. Mean *^{125}I-albumin clearance from isolated, ventilated and perfused rabbit lungs is inhibited by low temperature.*
Lungs were maintained either at 37 °C (*red*), 22 °C (*green*) or 4 °C (*blue*) and 60 min after establishing a steady-state equilibrium, ^{125}I-albumin was deposited into the alveolar space by nebulization. Elimination of the tracer from the lungs was monitored over 120 min. Counts were set at 100 % immediately after nebulization of ^{125}I-albumin tracer into the lungs. Each data point represents the mean of at least six independent experiments. For clarity, standard deviations have been omitted, however, they are incorporated into analyzes of these data in Figure 9.

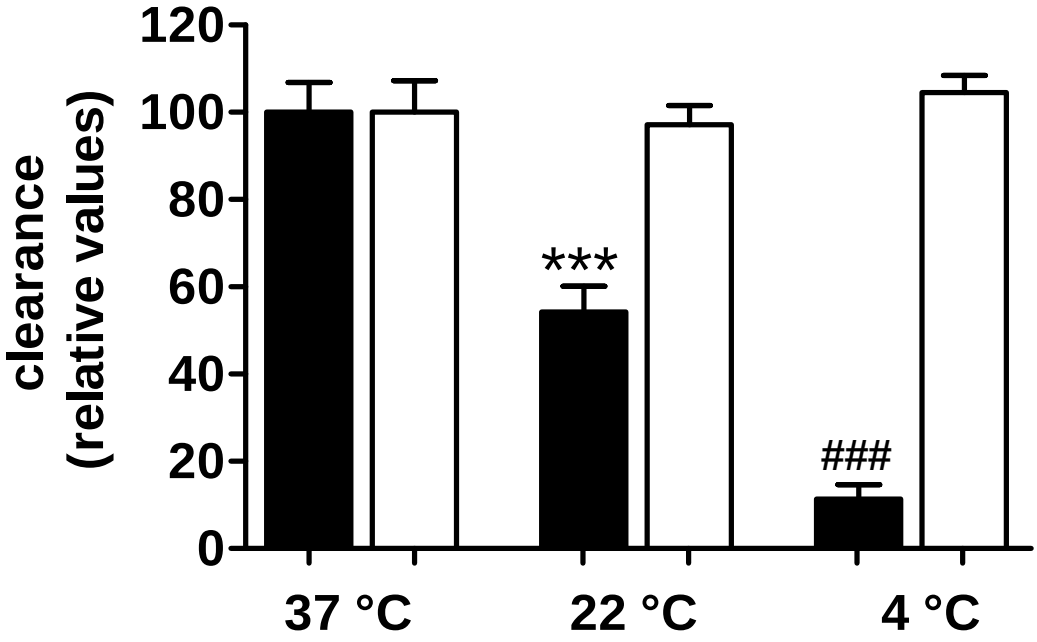

Figure 9. *Low temperature blocks albumin transport without affecting passive paracellular epithelial permeability of intact lungs.*
^{125}I-albumin transport (*solid bars*) was quantified from the data presented in Figure 8, as described in the "Materials and Methods" section. The ^{125}I-albumin transport in lungs maintained at 37 °C was set at 100%, while albumin transport in lungs maintained at low temperature was expressed relative to this control value. Passive [^{3}H]mannitol flux (*open bars*) was monitored by scintillation counting of perfusate samples taken at timed intervals after nebulization of the [^{3}H]mannitol tracer into the lungs, as described in "Materials and Methods". The passive [^{3}H]mannitol flux in lungs maintained at 37 °C was set at 100 %, while mannitol flux in lungs maintained at low temperature was expressed relative to this control value. Data represent the mean ± SD (n = 6 for all groups); ***, $p < 0.001$, compared to control; ###, $p < 0.001$, compared to control, or to lungs maintained at 22 °C.

3.2 Radio-labeled albumin nebulized into isolated rabbit lungs remains intact in the alveolar space over the course of the experiment

To assess the intactness of ^{125}I-albumin during the experimental time-course TCA precipitation was employed. The amount of ^{125}I-albumin in aliquots which were not nebulized but had been designed for aerosolization was found to be 95.4 ± 0.7 % intact (**Figure 10**). Similarly, after nebulization the bulk of radio-labeled albumin was intact in the alveolar space (87.6 ± 2.0 %) as measured from BAL samples directly after nebulization. The amount of intact radio-labeled albumin remained unchanged throughout the experiment, since 86.9 ± 3.4 % of the ^{125}I-albumin was TCA-precipitated at the end of the experiments (**Figure 10**). Intactness of ^{125}I-albumin was also assessed by centrifugal filter fractionation in samples obtained the same way described above. When a centrifugal filter device with a molecular cut-off of 50 kDa was used, results obtained were almost identical to those obtained by TCA precipitation. Before nebulization, 93.2 ± 1.2 % of ^{125}I-albumin was intact, while directly after nebulization, 89.2 ± 1.6 % and by the end of the experiment 87.8 ± 2.1 % of the radio-labeled albumin remained intact.

3.3 Transport of albumin between the alveolar and vascular space of isolated lungs is uni-directional

To address the question of whether albumin transport across the alveolo-capillary barrier was uni-directional (from the alveolar to the vascular compartment) or bi-directional, we administered FITC-albumin to the vasculature, and the flux of FITC-albumin into the alveolar space was measured. Experiments were performed either at 37 °C or at 4 °C, and concentrations of the FITC-albumin in the ELF and the perfusate were measured as described in detail in the “Materials and Methods” section. While approximately 30 % of the ^{125}I-albumin deposited into the alveolar space was cleared over the time-course of the experiment (120

min) at 37 °C (**Figure 8**), only 6.0 ± 1.1 % of the FITC-albumin applied to the vasculature appeared in the alveolar space over the same time-course at 37 °C (**Figure 11**).

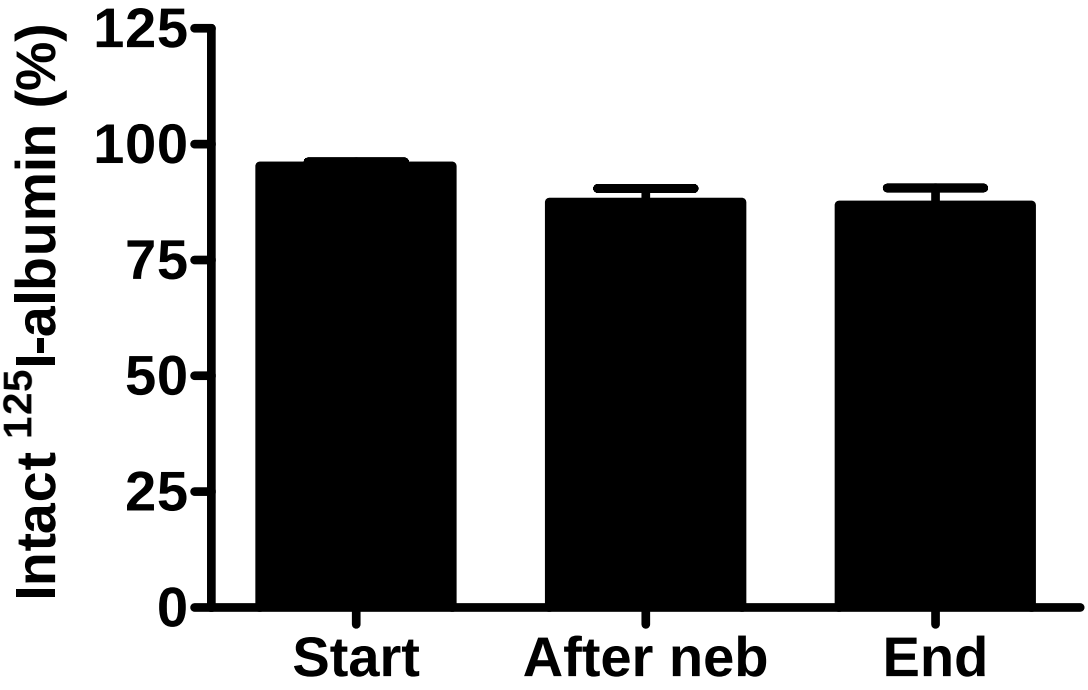

Figure 10. *^{125}I-albumin in the alveolar space remains intact throughout the experiment*
Intactness of ^{125}I-albumin was assessed by TCA precipitation before (*Start*), directly after nebulization (*After neb*), and at the end of the experiment (E*nd*). Data represent the mean ± SD (n = 6 for all groups).

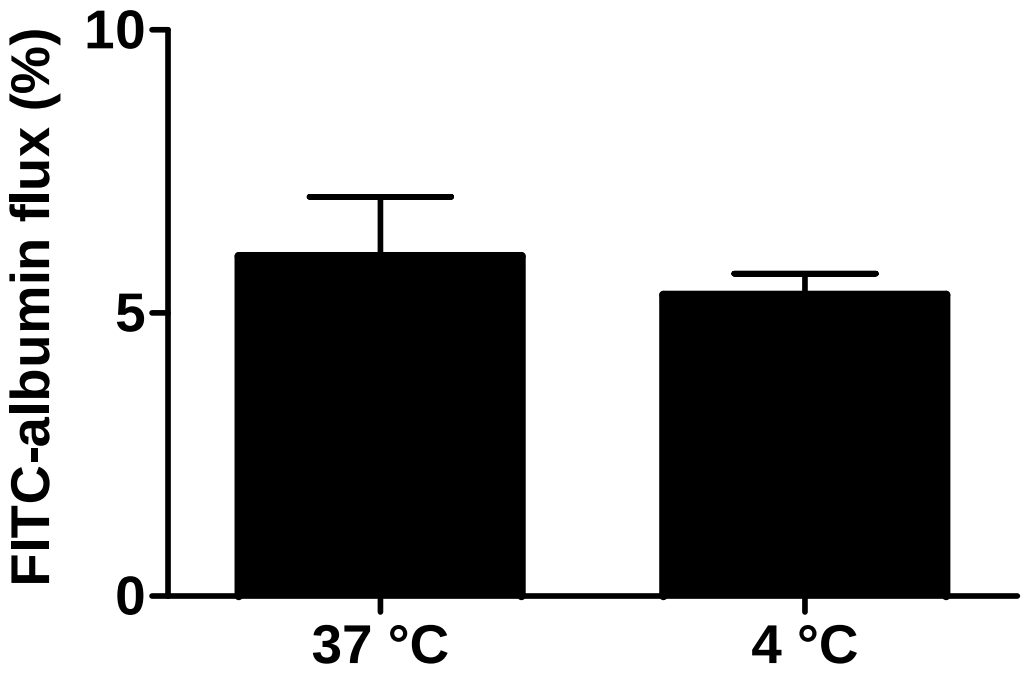

Figure 11. *Transport of albumin accross the alveolo-capillary barrier is uni-directional.*
The FITC-albumin was administered to the perfusate and quantified in the ELF from BAL samples. Experiments were conducted at 37 °C or 4 °C. The FITC-albumin concentration in the perfusate was set to 100 %, and the concentration of FITC in the BAL was calculated as a percentage of the FITC concentration in the perfusate. Bars represent the mean ± SD (n = 6 for all groups).

When experiments at 4 °C were performed, nearly identical transport rate of FITC-albumin from the vascular to the alveolar space was measured (5.3 ± 0.5 %) (**Figure 11**), suggesting that the bulk of the FITC-albumin movement was probably mediated by passive paracellular processes. In line with these findings, when ^{125}I-albumin transport from the alveolar to the vascular compartment was measured at 4 °C, a similar clearance rate (3.7 ± 0.4 %), compared to 100 % in the perfusate, was calculated (**Figure 8**). These data collectively support the hypothesis that movement of albumin through the alveolo-capillary barrier was predominantly uni-directional, occuring *from* the alveolar *to* the vascular space.

3.4 Albumin is taken up intact by the alveolar epithelium of isolated rabbit lungs

In further experiments which were designed to clarify whether albumin was degraded by proteases in the ELF, or taken up intact by the alveolar epithelium, we aerosolized protease inhibitors to the distal air spaces to inhibit protease activity. We applied EDTA for metallo-proteases (Chiancone, Thulin et al. 1986) as well as AEBSF for the inhibition of serine protease activity (Lunn and Sansone 1994). Serine- and metallo-proteases represent the main source of protease activity in the alveolar space (Gross 1995; Greenlee, Werb et al. 2007). When clearance of ^{125}I-albumin from the alveolar space was measured after pre-nebulization of AEBSF, transit of ^{125}I-albumin across the alveolo-capillary barrier was unchanged when compared to control lungs that have been pre-nebulized with physiological saline (30.2 ± 2.2 % and 30.4 ± 1.8 %, respectively; **Figure 12**). Similarly, pre-nebulization of EDTA did not change the rate of movement of the radio-labeled protein either (30.7 ± 1.4 %; **Figure 12**). These results suggested to us that degradation activity, mediated by serine and metallo proteases in the ELF, had no effect on albumin transport across the alveolo-capillary barrier.

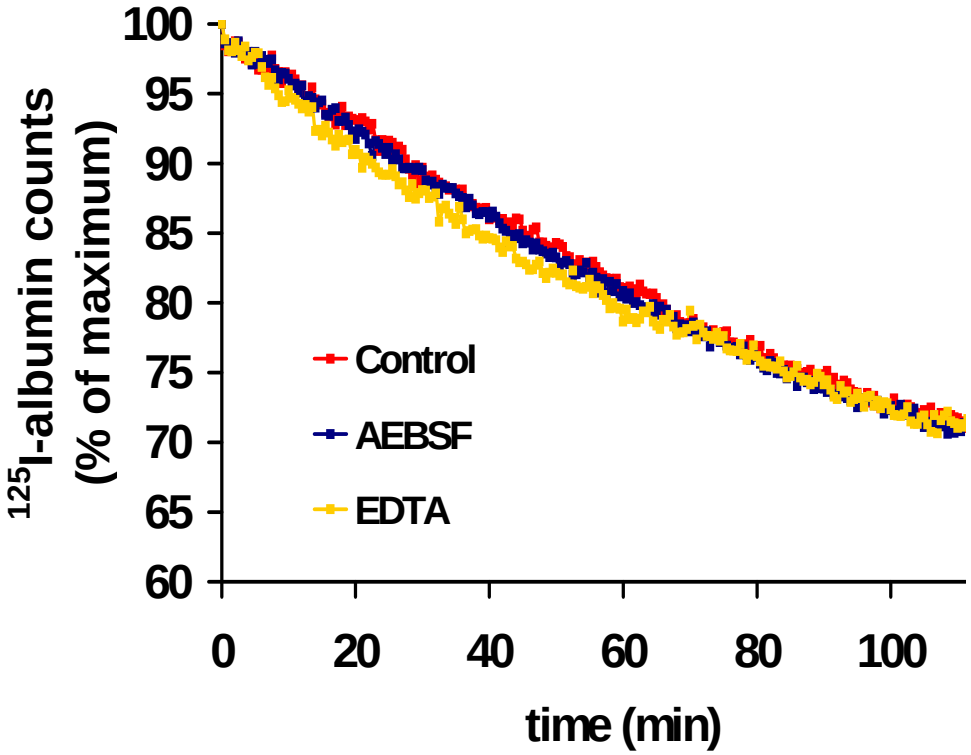

Figure 12. *Active clearance of ^{125}I-albumin from the alveolar space is not affected by metallo- and serine proteases.*
Control lungs (*red*) were maintained at 37 °C and sham-nebulized with 0.1 % [vol/vol] DMSO in physiological saline 30 min after establishing steady-state equilibrium. In additional experiments, instead of sham-nebulization, AEBSF (*blue*) or EDTA (*yellow*) was nebulized to lungs and after a further 30 min, ^{125}I-albumin tracer was deposited into the alveolar space, and elimination of this tracer from the lung was monitored as described in the legend to Figure 8. Each data point represents the mean of six independent experiments.

3.5 Albumin movement across the alveolo-capillary barrier is a saturable, active process

In some experiments, lungs were pre-nebulized with a 1000-fold molar excess of unlabeled albumin (compared to the radioactive tracer) prior to aerosolization of ^{125}I-albumin to investigate whether transit of the radio-labeled protein can be inhibited by competition. Deposition of the excess native albumin into the alveolar space significantly decreased the active transport of ^{125}I-albumin from the air space to the vascular compartment of the lung, since the rate of active transport of the tracer was inhibited by 31.6 ± 1.2 % compared to control lungs that had been pre-nebulized with physiological saline (**Figure 13 A and B**).

Under normal conditions, the perfusate does not contain any protein, and thus an albumin gradient between the two sides of the alveolo-capillary barrier occurs. In additional studies, when excess unlabeled albumin was administered to the perfusate to eliminate this oncotic gradient between the alveolar and the vascular compartments, no effect on ^{125}I-albumin transit was observed (**Figure 13 A and B**) further suggesting that passive movement of the radio-labeled protein was not a significant contributor to albumin clearance from the air space. These data indicate that the vast majority of the transepithelial albumin movement is mediated by active transport processes.

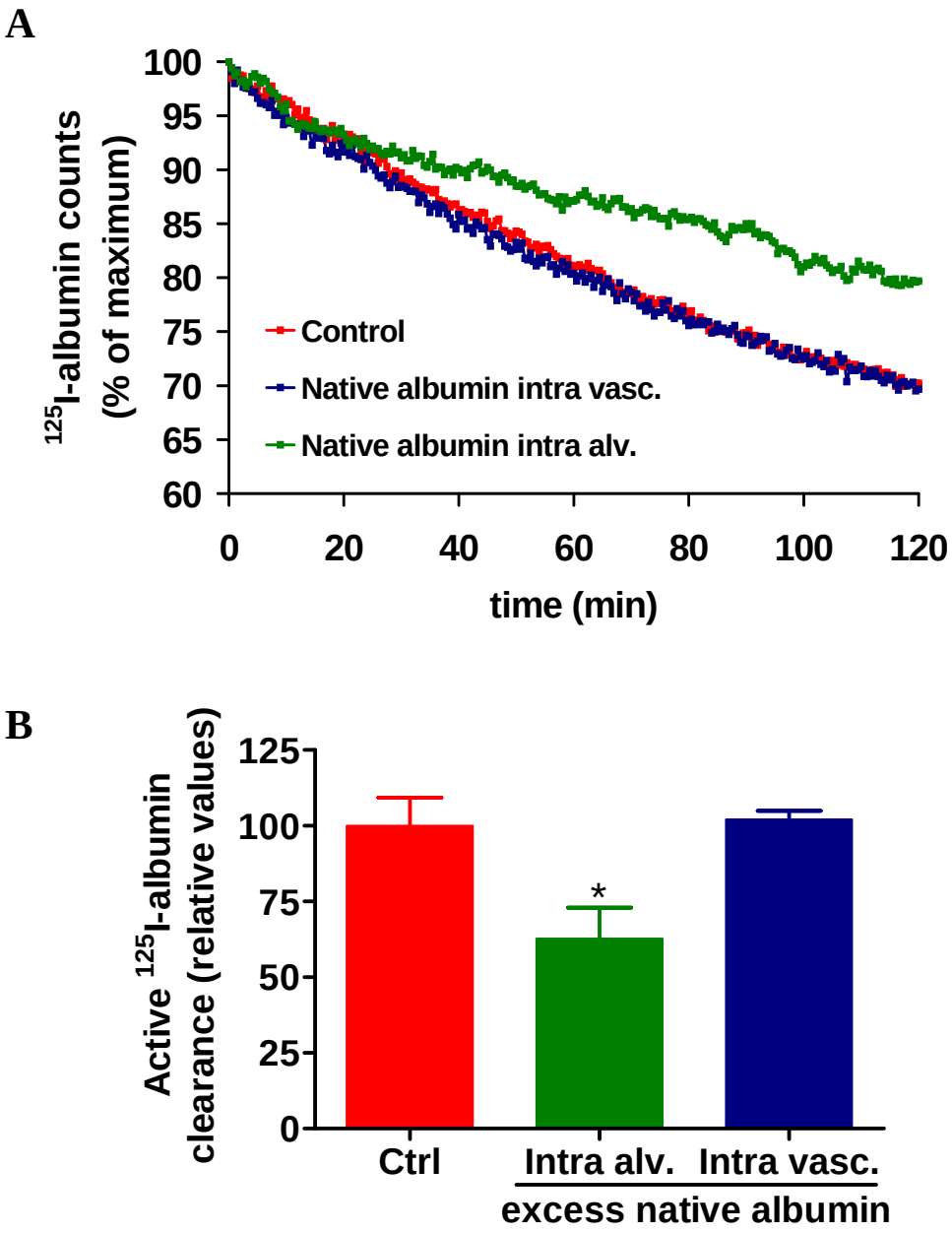

Figure 13. *Excess intra-alveolar (but not intravascular) native albumin inhibits ^{125}I-albumin clearance from intact rabbit lungs.*

(A) Control lungs (*red*) were maintained at 37 °C and sham-nebulized with physiological saline 30 min after establishing a steady-state equilibrium. In some experiments, instead of sham-nebulization, a 1000-fold molar excess of native albumin was nebulized into the lungs (*green*). Alternatively, excess native albumin (20 mg/ml) was applied to the perfusate of the lungs (*blue*) after establishing a steady-state equilibrium. After a further 30 min, ^{125}I-albumin tracer was nebulized to the lungs, and elimination of this tracer from the lung was monitored over 120 min, as described in the legend to Figure 7. (B) Active ^{125}I-albumin transport was set at 100% in control lungs and active transport of the tracer in excess native albumin-treated lungs was expressed relative to this control value. Bars represent the mean ± SD (n = 6 for all groups); *, $p < 0.05$.

3.6 Albumin is taken up by A549 cells via an active process

To investigate whether albumin was taken up actively by the mammalian epithelium, A549 monolayers were pre-treated with a 1000-fold molar excess of unlabeled albumin (compared to the radioactive tracer), prior to ^{125}I-albumin administration, to assess the effect of competition. In control experiments, uptake of ^{125}I-albumin was found to be bi-exponential, where a rapid initial phase (typically in the first 10 min) was followed by a slower uptake rate later (between 10 and 60 min), with an area under the curve (AUC) of 91.4 ± 7.1 nCi/h (**Figure 14**). Pretreatment with the native albumin markedly inhibited the uptake of the radio-labeled tracer (AUC 23.2 ± 3.4 nCi/h; **Figure 14**), indicating a competitive antagonistic effect of the unlabeled albumin on ^{125}I-albumin uptake. Thus the γ-radiation that occurred in the presence of the 1000-fold molar excess of unlabeled albumin was considered as binding of ^{125}I-albumin to the cell surface, and the difference between this and the control curve was considered as the active ^{125}I-albumin uptake.

3.7 Albumin movement from the alveolar compartment to vasculature involves caveolae-mediated endocytosis

To address the question of whether caveolae-mediated endocytosis was involved in the movement of ^{125}I-albumin from the air space to the vasculature, NEM (Schnitzer, Allard et al. 1995) or filipin (Schnitzer, Oh et al. 1994) were administered to the vascular compartment of the isolated lung prior to the nebulization of ^{125}I-albumin. Application of NEM resulted in a significant inhibition of the transit rates of the radio-labeled protein from the distal air space (36.7 ± 5.4 %; **Figure 15 A and B**). Similarly, when filipin was administered to the perfusate prior to ^{125}I-albumin aerosolization the active component of the tracer transit was blocked by 31.6 ± 4.8 % (**Figure 15 A and B**). These data

suggested that caveolae-mediated endocytosis plays a role in albumin movement from the alveolar to the vascular compartment.

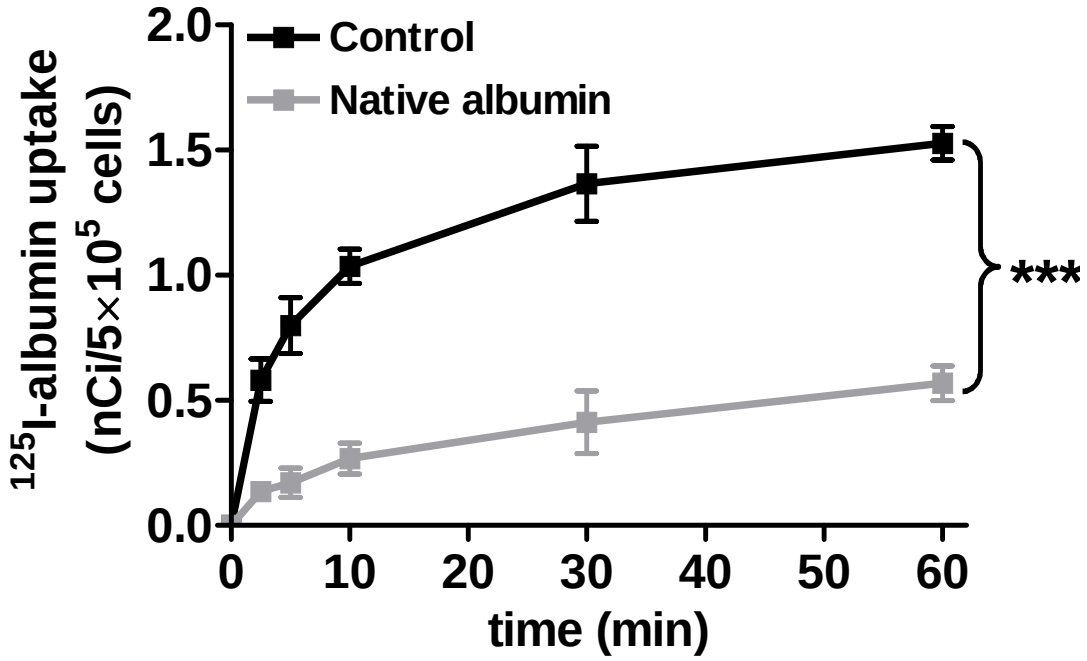

Figure 14. *Uptake of ^{125}I-albumin by human epithelial A549 cells was blocked by excess native albumin.*
Subconfluent monolayers of A549 cells plated in 6-well tissue culture dishes were pre-incubated for 30 min with 2 ml of DPBS-G in the absence or presence of excess native albumin. The media was then removed and 2 ml of DPBS-G containing ^{125}I-albumin was applied to the cells and uptake of the radio-labeled protein was terminated after 2.5, 5, 10, 30 or 60 min from cell lysates by gamma-emission counting. Each data point represents the mean of at least four independent experiments. ***, $p < 0.001$, compared to control.

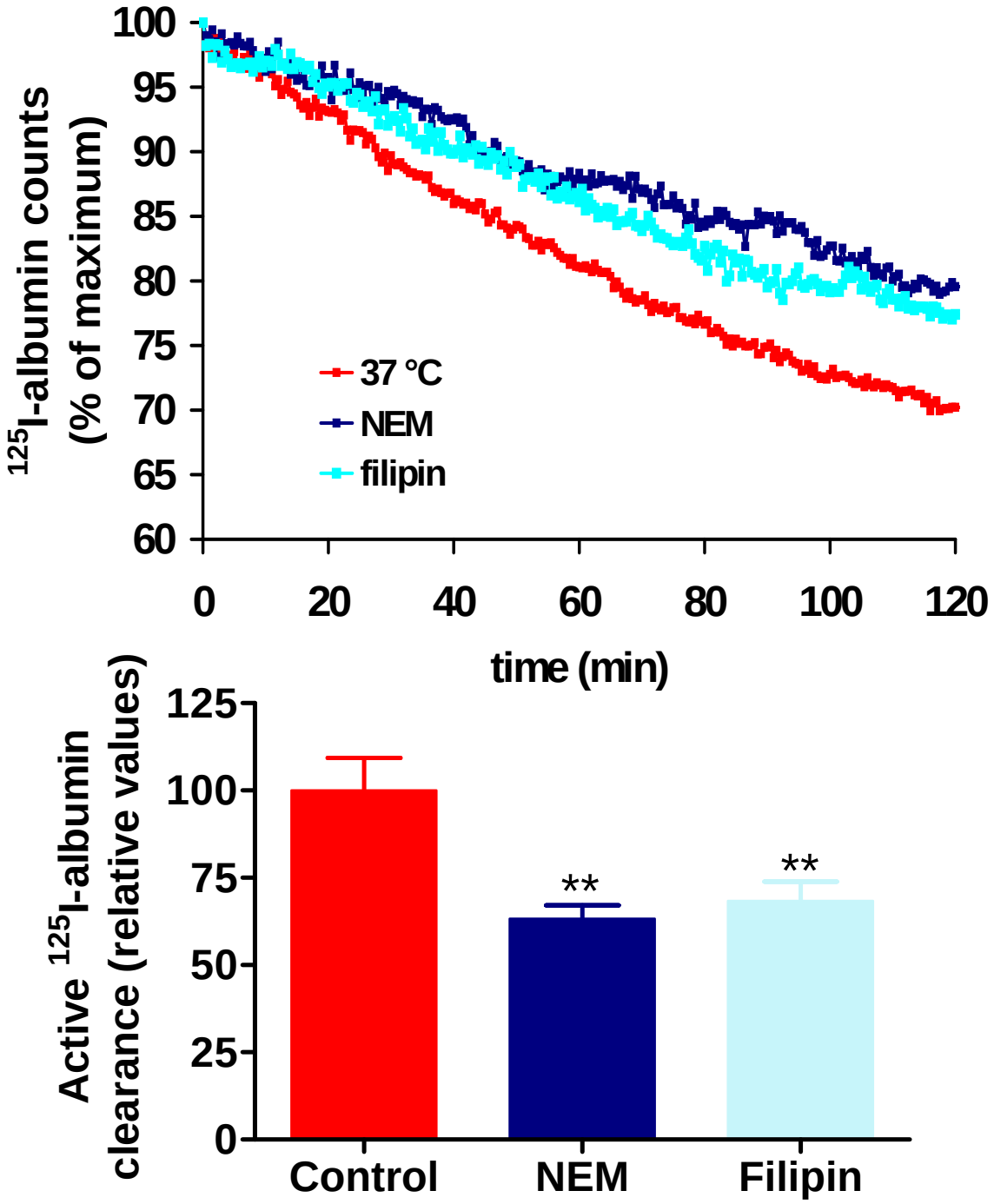

Figure 15. *Caveolae-mediated endocytosis is required for active clearance of ^{125}I-albumin from the alveolar space to the vascular compartment.*
(A) Thirty minutes after achieving steady-state, lungs were either sham-treated (*red*) or were treated with NEM (*blue*) or filipin (*bright blue*) in 0.1 % (vol/vol) DMSO instead of the sham application. After a further 30 min, ^{125}I-albumin tracer was deposited into the alveolar space, and elimination of this tracer from the lung was monitored as described above. Each data point represents the mean of six independent experiments. (B) Active ^{125}I-albumin transport was set at 100% in control lungs and active transport of the tracer in caveolae-mediated endocytosis inhibitor-treated lungs was expressed relative to this control value. Values represent the mean ± SD (n = 6 for all groups); **, $p < 0.01$.

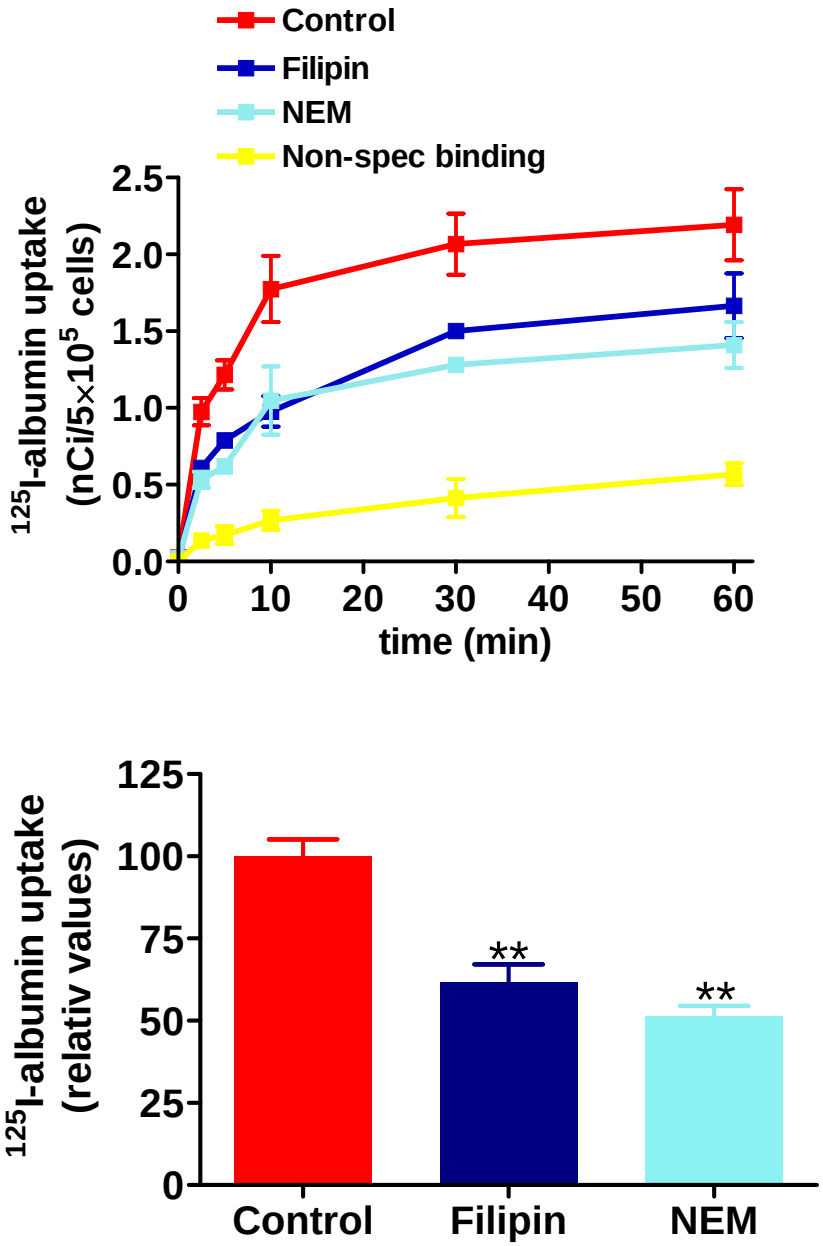

Figure 16. *^{125}I-albumin uptake by A549 cells is mediated by caveolae-mediated endocytosis.*

(A) Subconfluent monolayers of A549 cells plated in 6-well tissue culture dishes were pre-incubated for 30 min either with filipin (*blue*) or *N*-ethylmaleimide (*bright blue*) or their vehicle. The medium was removed and replaced with medium containing ^{125}I-albumin in the presence of the inhibitor or vehicle. The uptake of the radio-labeled protein was determined after 2.5, 5, 10, 30 or 60 min from cell lysates by gamma-emission counting. Each data point represents the mean ± SD of at least four independent experiments. (B) ^{125}I-albumin uptake was expressed as area under the curve and compared to vehicle-treated controls which were set at 100 % **, $p < 0.01$, when compared to control.

3.8 Albumin uptake by A549 cells requires caveolae function

To further investigate whether caveolae function was necessary for the alveolar epithelial uptake of albumin, this process was assessed in A549 cells. In the presence of filipin or NEM, the uptake of ^{125}I-albumin by A549 cells was inhibited to a similar extent as was noted in the isolated lung model. These results are summarized in **Figure 16 A and B**. These data suggested that caveolae-mediated endocytosis was an important element of albumin uptake by A549 cells.

3.9 Albumin movement from the alveolar compartment to vasculature includes clathrin-dependent endocytosis

In additional experiments we further investigated the mechanisms of endocytosis that might mediate the transport of ^{125}I-albumin from the alveolar compartment to the vasculature, and assessed a role for clathrin-dependent endocytosis. When the clathrin-mediated endocytosis inhibitor, phenylarsine oxide (Visser, Stevanovic et al. 2004), was nebulized into the alveolar space, a significant decrease in active ^{125}I-albumin transit from the alveolar epithelium to the vascular compartment of the lung was evident, since the rate of ^{125}I-albumin active transport was inhibited by 30.2 ± 1.2 % (compared to control lungs that had been pre-nebulized with physiological saline; **Figure 17 A and B**). Additionally, in further experiments, another clathrin-dependent endocytosis inhibitor, chlorpromazine (Wang, Rothberg et al. 1993), was administered to the air spaces prior to aerosolization of ^{125}I-albumin to confirm the findings with phenylarsine oxide. Application of chlorpromazine, similarly to what was observed with phenylarsine oxide, resulted in a clear decrease (33.1 ± 2.2 %, compared to control lungs treated with vehicle only; **Figure 17 A and B**) in the transport rate of aerosolized ^{125}I-albumin from the alveolar compartment to the vasculature.

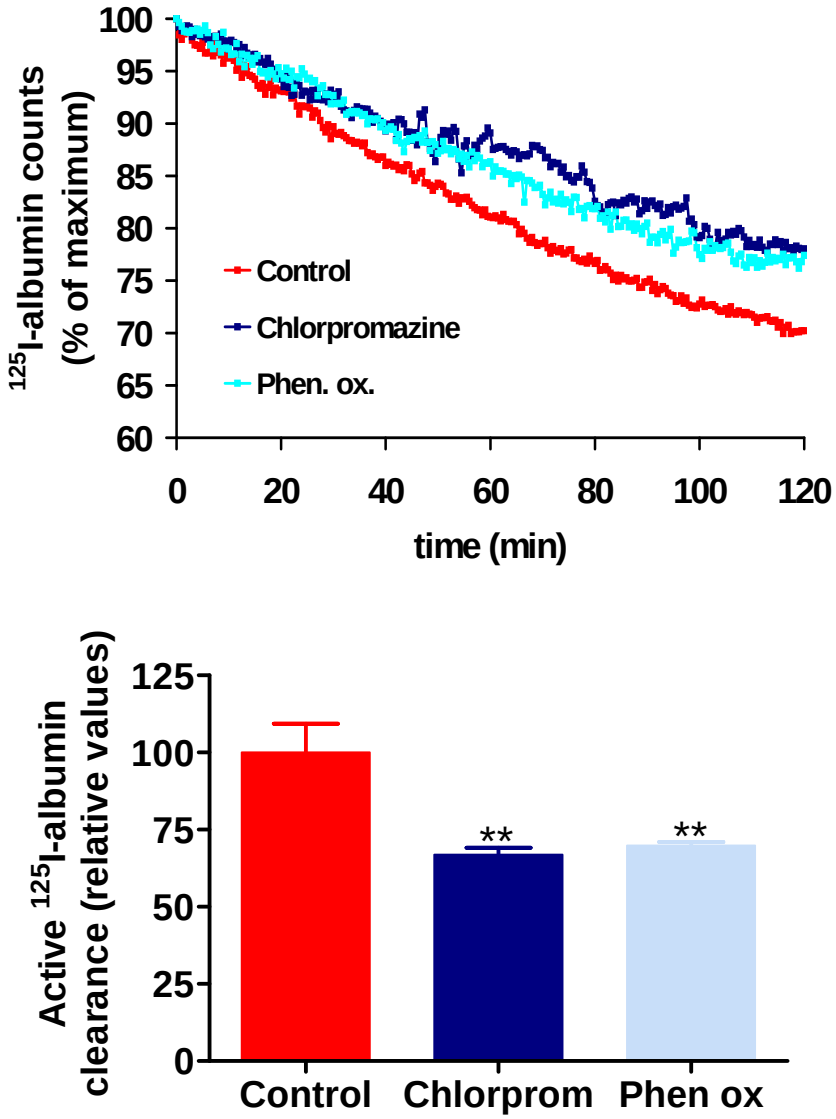

Figure 17. *Clathrin-dependent endocytosis is required for the active clearance of ^{125}I-albumin from the alveolar space.*
(A) Identical to experiments illustrated in Figure 7, control lungs were sham-nebulized with 0.1 % [vol/vol] DMSO in physiological saline (*red*) 30 min after achieving steady-state. In additional experiments, lungs were either aerosolized with chlorpromazine (Chlorprom, *blue*) or with phenylarsine oxide (Phen ox, *bright blue*) instead of the sham-nebulization. ^{125}I-albumin tracer was deposited into the alveolar space after a further 30 min and elimination of the tracer from the lung was monitored over 120 min. Each data-point represents the mean of six independent experiments. (B) Active ^{125}I-albumin transport was set at 100% in control lungs and active transport of the tracer in clathrin-dependent endocytosis inhibitor-treated lungs was expressed relative to this control value. Bars represent the mean ± SD (n = 6 for all groups); **, $p < 0.01$.

3.10 Albumin uptake in A549 cells involves clathrin-dependent endocytosis

To address the role of clathrin-dependent endocytosis in ^{125}I-albumin uptake by A549 cells, two clathrin-dependent endocytosis inhibitors, phenylarsine oxide (Visser, Stevanovic et al. 2004) and chlorpromazine (Wang, Rothberg et al. 1993), were applied separately to the cell monolayer. In both experimental groups, ^{125}I-albumin uptake was markedly impaired by these inhibitors as expressed by the decrease in the AUC (63.9 ± 4.1 % and 63.2 ± 5.9 %, respectively) compared to uptake assessed in control experiments, where vehicle only was applied (**Figure 18 A and B**). These data suggested that clathrin-dependent endocytosis was required for albumin uptake by A549 cells.

3.11 Albumin uptake in A549 cells requires both caveolae-mediated and clathrin-dependent endocytic processes

Since both caveolae- and clathrin-dependent endocytosis appeared to be important for albumin uptake by the alveolar epithelium, the combined role of these two endocytic processes was assessed by simultaneously applying inhibitors of both processes. Therefore, we applied the clathrin inhibitor, chlorpromazine, and the caveolae inhibitor filipin, together or separately. Consistent with the previous findings, both chlorpromazine and filipin significantly inhibited ^{125}I-albumin uptake in A549 cells (**Figure 19 A and B**). Importantly, when these inhibitors were applied in combination, a significantly greater inhibition occurred than did after the drugs were administered separately (**Figure 19 A and B**). These effects were not due to cytotoxicity of any of the drugs applied. Under control conditions, approximately 1-2 % of cells were stained by the Trypan Blue dye, representing dead cells. None of the pharmacological inhibitors had any effect on cell viability, since the amount of stained cells did not exceed 2 % under any experimental condition.

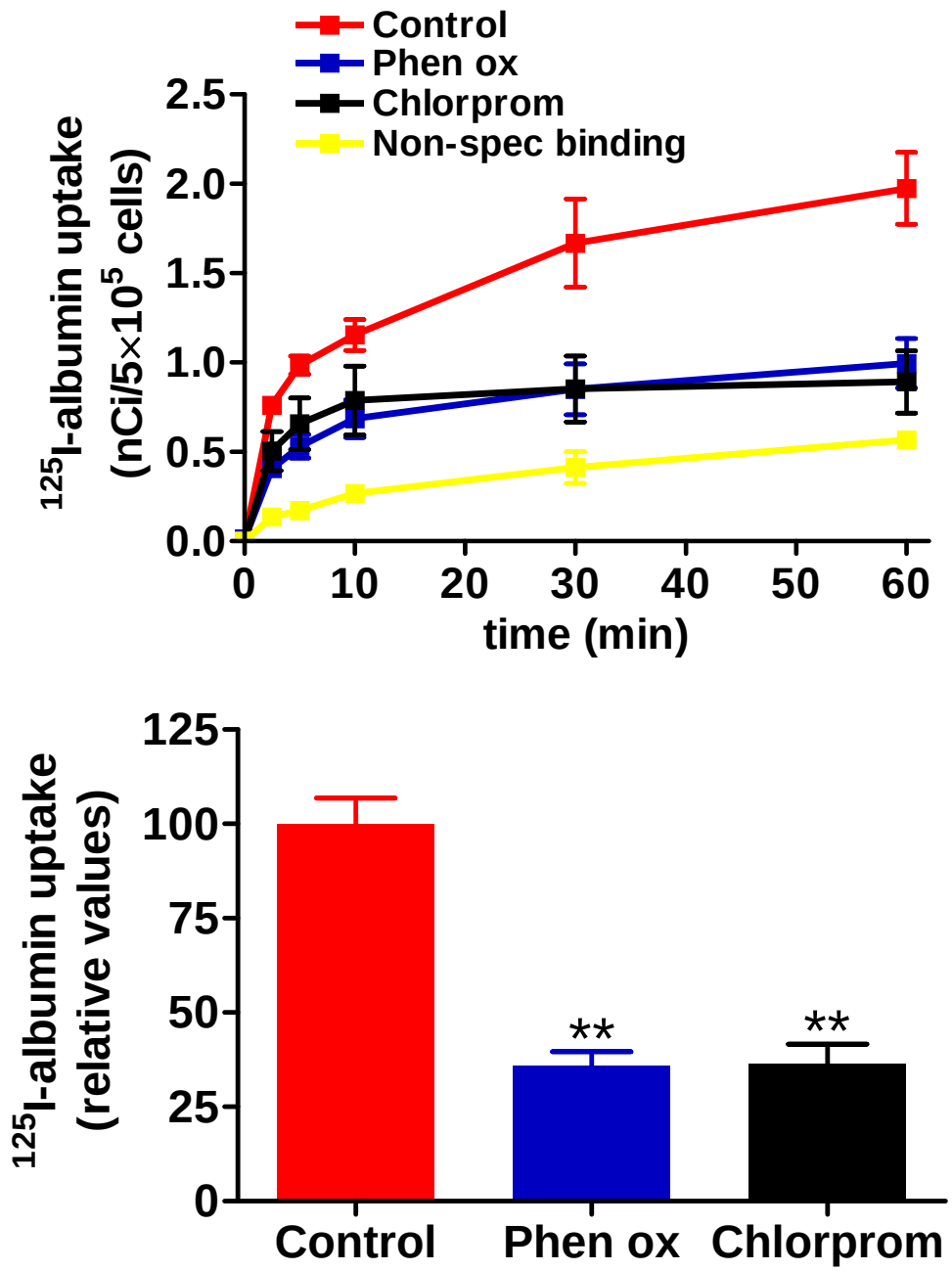

Figure 18. *^{125}I-albumin uptake by A549 cells is mediated by clathrin-mediated endocytosis*

(A) Subconfluent monolayers of A549 cells plated in 6-well tissue culture dishes were preincubated for 30 min either with phenylarsine oxide (Phen ox, *blue*) or chlorpromazine (*black*) or their vehicles (*red*). The medium was then removed and replaced with medium containing ^{125}I-albumin in the presence or absence of the inhibitors. Uptake of the radio-labeled protein was determined after 2.5, 5, 10, 30 or 60 min from cell lysates by gamma-emission counting. Each data point represents the mean of at least four independent experiments. (B) ^{125}I-albumin uptake was expressed as area under the curve and compared to vehicle-treated controls which were set at 100 % **, $p < 0.01$, when compared to control.

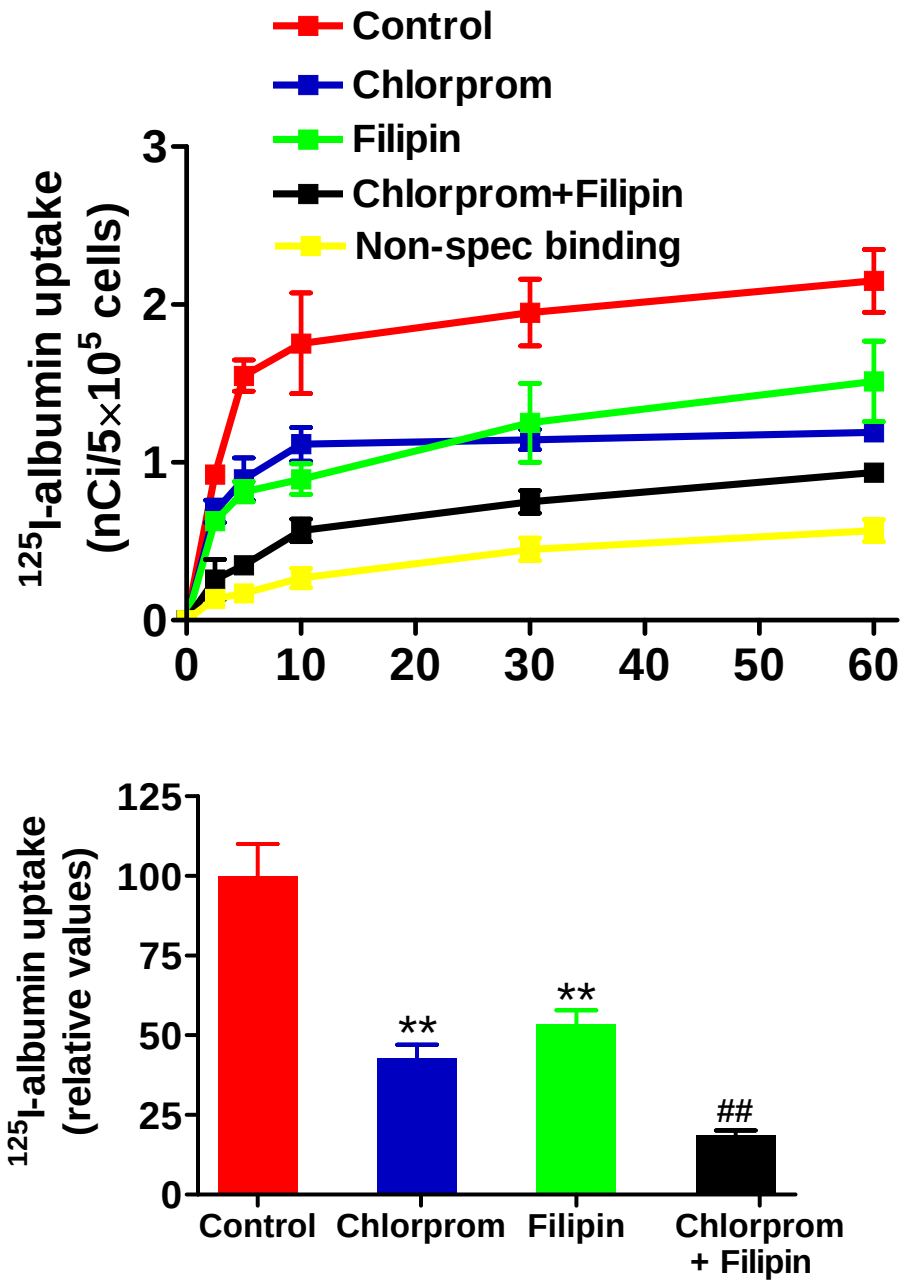

Figure 19. *^{125}I-albumin uptake by A549 cells is mediated by clathrin-mediated endocytosis*

(A) Subconfluent monolayers of A549 cells plated in 6-well tissue culture dishes were pre-incubated for 30 min either with vehicle (*red*), chlorpromazine (Chlorprom, *blue*) or filipin (*green*) or their combinations (*black*). The medium was then removed and replaced by medium containing ^{125}I-albumin in the presence or absence of the inhibitors. Uptake of the radio-labeled protein was determined after 2.5, 5, 10, 30 or 60 min from cell lysates by gamma-emission counting. Each data point represents the mean of at least four independent experiments. (B) ^{125}I-albumin uptake was expressed as area under the curve and compared to vehicle-treated controls which were set at 100 %. **, $p < 0.01$, when compared to control; ##, $p < 0.01$, when compared to any other group.

3.12 Albumin movement across the alveolo-capillary barrier is mediated by transcytosis

In further experiments, phalloidin oleate, a membrane-permeable inhibitor of actin cytoskeletal rearrangement (Stenbeck and Horton 2004), and thus transcytosis, was pre-nebulized into lungs prior to aerosolization of ^{125}I-albumin, to investigate whether transport of the radio-labeled protein can be altered by inhibition of this process. Deposition of phalloidin oleate into the ELF significantly decreased the active transport of the radio-labeled protein from the alveolar space to the vasculature of the lung (**Figure 20 A and B**), since the active transport rate of ^{125}I-albumin was inhibited by 51.4 ± 1.2 % compared to control lungs that had been pre-nebulized with vehicle alone. In some additional experiments, monensin, another potent inhibitor of transcytosis (Sakagami, Byron et al. 2002), was deposited by nebulization into the air space, followed by aerosolization of ^{125}I-albumin, to confirm our findings with PO. Indeed, similar to the effects observed with PO, ^{125}I-albumin transport across the alveolo-capillary barrier significantly decreased after monensin treatment, since the rate of active radio-labeled albumin transit was blocked by 34.0 ± 1.2 % (compared to control lungs; **Figure 20 A and B**). These data collectively suggest that transcytosis plays an important role in movement of albumin from the distal air spaces to the vasculature.

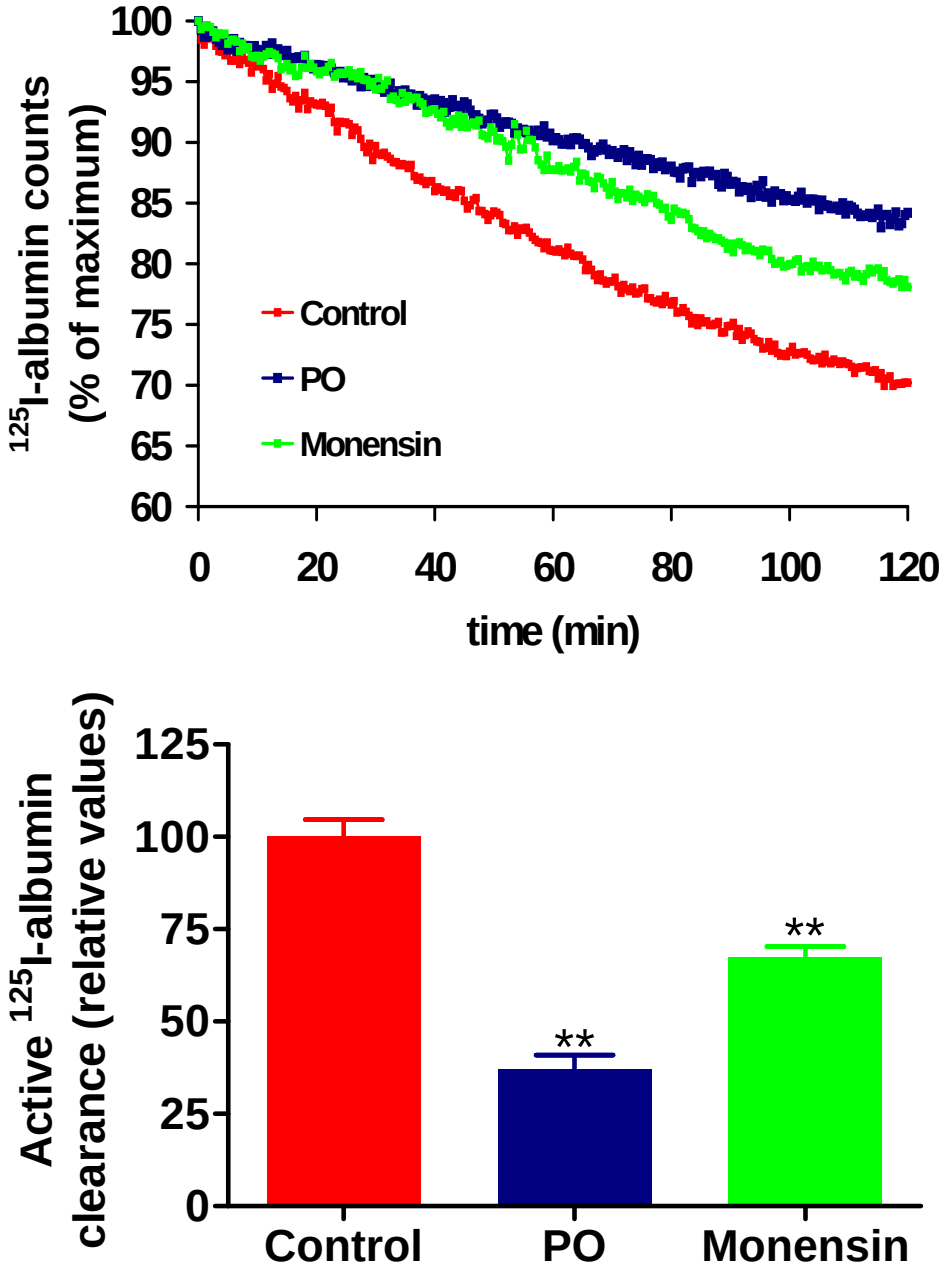

Figure 20. *Active clearance of ^{125}I-albumin from the alveolar space is mediated by transcytosis of the protein.*
(A) Control lungs were sham-nebulized with 0.1 % [vol/vol] methanol in physiological saline (*red*) 30 min after achieving steady-state. In additional experiments, lungs were either aerosolized with phalloidin oleate (PO, *blue*) or with monensin (*green*) instead of the sham-nebulization. The ^{125}I-albumin tracer was deposited into the alveolar space after a further 30 min and elimination of the tracer from the lung was monitored as described above. Data represent the mean of six independent experiments. (B) Active ^{125}I-albumin transport was set at 100% in control lungs and active transport of the tracer in transcytosis inhibitor-treated lungs was expressed relative to this control value. Bars represent the mean ± SD (n = 6 for all groups); **, $p < 0.01$.

3.13 Albumin movement across the alveolo-capillary barrier involves albumin degradation

Since inhibition of transcytosis (which by definition mediates transit of intact macromolecules) resulted only in a partial blockage of albumin movement across the air-blood barrier, it was speculated that some of the radio-labeled protein may be degraded during the transport process. Therefore, in further experiments, we assessed the amount of intact ^{125}I-albumin in the vascular space at the end of the experiment by TCA precipitation. At 37 °C approximately 50 % of the radio-labeled albumin was found to be intact (**Figure 21**). In contrast, in experiments conducted at 4 °C, ~90 % of the vascular ^{125}I-albumin was degraded, however, the amount of radiation in the perfusate was only ~10 % that oberved in those experiments conducted at normal body temperature (**Figure 21**). Intactness of ^{125}I-albumin was also assessed by centrifugal filter fractionation in samples obtained the same way described above. When a centrifugal filter device with a molecular cut-off of 50 kDa was used, results obtained were almost identical to those obtained by TCA precipitation (data not shown). The potential significance of these findings is discussed in detail in the “Discussion” section.

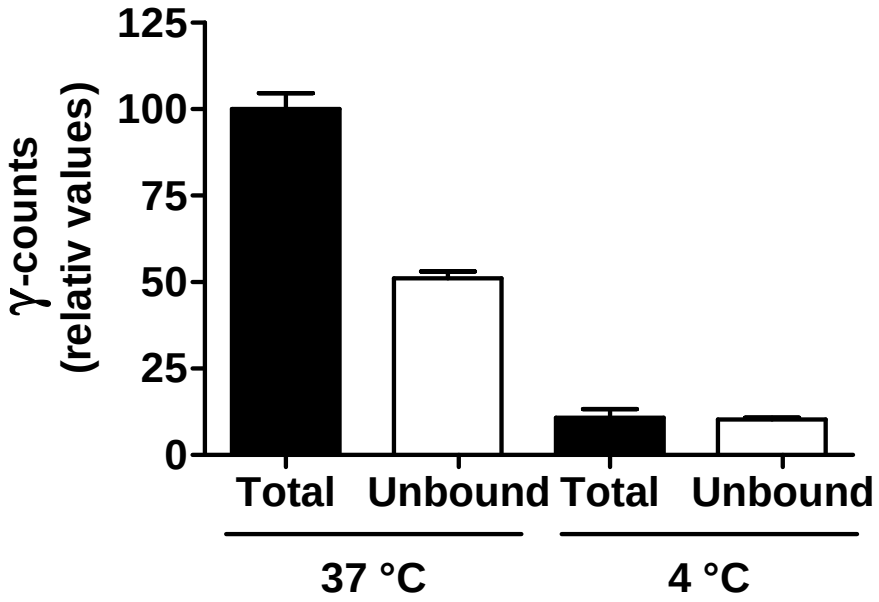

Figure 21. *^{125}I-albumin is partially degraded in the vascular space.*
Intactness of ^{125}I-albumin was assessed by TCA precipitation from perfusate samples from experiments conducted either at 37 °C or at 4 °C. Data are given as mean ± SD and represent relative values compared to total radiation of perfusate samples from experiments conducted at 37 °C (*solid bar on the left*). The *solid bar on the right* represents the total radiation of perfusate samples at 4 °C. *Open bars* represent free ^{125}I released from degraded radio-labeled albumin. n = 6 for all groups.

4. Discussion

Alveolar protein balance is an emerging area of interest in the pathogenesis of ARDS (Hastings, Folkesson et al. 2004). Excess alveolar albumin in particular, has been implicated in the pathogenesis of ARDS, and has been identified as a prognostic factor for this syndrome (Bachofen and Weibel 1977). Levels of plasma proteins are elevated in the alveolar fluids from patients with ARDS, contributing to the persistence of pulmonary edema and thereby impeding healing, and thus clearance, of the excess alveolar protein which is a key step in the resolution of acute lung injury (Hastings, Folkesson et al. 2004). Over the past decades several possible mechanisms of alveolar protein clearance have been studied, including mucociliary clearance, phagocytosis by macrophages, degradation of proteins, passive paracellular transport and active transcellular transport across the epithelium (Folkesson, Matthay et al. 1996; Kim and Malik 2003; Hastings, Folkesson et al. 2004). However, despite intensive research, the precise mechanism by which proteins are cleared from the distal air spaces remains poorly understood.

We addressed this question in an isolated, ventilated, and perfused rabbit lung model. The main advantage of this intact organ model is that the (patho)physiology of the lung can be studied independently of the rest of the organism; local physiological hemostasis, physiological cell-to-cell contacts and cell polarity are maintained, hemodynamics are controlled, and relevant physiological parameters can be measured (Seeger, Walmrath et al. 1994). However, the lung isolation procedure requires neural dissection and perfusion and artificial respiration, which may alter biochemical or metabolic processes of the lung, thereby limiting the use of this model. Furthermore, in spite of thorough rinsing of the vasculature of the isolated organ, adherent leukocytes including monocytes, granulocytes and lymphocytes remain in the vascular bed, and their contribution to physiological or biochemical events is unclear and difficult to control. In spite of these drawbacks, however, we do not believe that they call

into question the validity of the conclusions we draw from our investigations, since all experiments were performed in lungs which exhibited virtually identical steady-state conditions. Thus, the isolated, ventilated and perfused rabbit lung model represents a powerful tool with which to investigate movement of radio-labeled molecules (such as ^{125}I-albumin) in real-time from the alveolar to the vascular compartment or *vice versa*.

The complexity of the isolated lung model makes it difficult to ascribe physiological and biochemical events to specific cell types. Thus, to further confirm our hypothesis that excess albumin was taken up by the epithelium of the distal air spaces, experiments in cultured human alveolar epithelial A549 cells were performed. This cell line is a popularly-used *in vitro* model of type II alveolar epithelial cells (Foster, Oster et al. 1998). The A549 cell line exhibits many characteristics of alveolar type II cells, including the existence of lamellar inclusion bodies and the ability to synthesize surfactant constituents (Lieber, Smith et al. 1976). Furthermore, A549 cells were demonstrated to take up transferrin, a maker of receptor-mediated endocytosis, as well as cationised ferritin, an unspecific marker for absorption in a temperature-, time-, and concentration-dependent manner. This cell-line was also used as a tool to investigate sodium transport studies (Lazrak, Samanta et al. 2000) because of similarities in sodium channel expression when compared with primary alveolar type II cells. However, in contrast to type II cells, A549 cells neither polarize nor form fully-developed intercellular junctions, and are therefore unable to establish electrophysiologically tight monolayers (Foster, Oster et al. 1998). Consequently, transcytosis cannot be addressed in this cell-line. Additionally, A549 cells represent only one cell type of the alveolar epithelium (type II cells, which have been accredited with a key role in transport processes) but not type I cells. Nevertheless, A549 cells represent a powerful tool to with which investigate alveolar epithelial uptake of macromolecules such as albumin.

All previous reports agree that mucociliary clearance and phagocytosis by macrophages contribute marginally to the clearance of excess protein from the air spaces, whereas cleavage of proteins, and passive or active transport across the

epithelium, might be of importance in trafficking of proteins across the alveolar epithelium (Hastings, Folkesson et al. 2004). We first addressed the question of whether excess alveolar albumin was cleared by active or passive processes, and whether movement of proteins through the alveolo-capillary barrier was vectorial. We deposited ^{125}I-albumin into the distal air spaces of intact lungs by nebulization and measured movement of the radio-labeled albumin from the alveolar compartment to the vascular compartment in real-time using γ-detectors placed around the lungs and the perfusate reservoir.

When generating particles of alveolar-accessible size, as currently done, aerosolization is a most powerful tool to achieve homogeneous alveolar deposition of any solute. When this technique was used for labeled albumin, controls were performed to ascertain that the protein was not fragmented and that the label was not dissociated from the protein by the ultrasonic device. A yield of ~50 % of the total nebulized volume was deposited into the lung as calculated from the tracer measurement of lung and perfusate which compares favorably with data in the literature, in which deposition fractions ranging from <5 % up to 60 % are reported (Schlesinger 1985; Ghofrani, Kohstall et al. 2001; Vadasz, Morty et al. 2005). The reproducibility of the aerosol technique is well reflected by the small variation in counts detected after tracer loading between the different lungs. The use of highly sensitive γ detectors, constructed to capture a large percentage of the lung and perfusate reservoir surfaces, allowed highly reproducible data with relatively low absolute tracer quantities to be obtained.

Our data indicated that under control conditions (37 °C) approximately 30 % of ^{125}I-albumin deposited into the alveolar space was cleared during the 120 min time-course of the experiment (Figure 8). The rate of albumin movement across the alveolo-capillary barrier was thus somewhat more rapid than previously reported by our group (albumin passage after 120 minutes ~18 %) (Ghofrani, Kohstall et al. 2001) for isolated, ventilated and perfused rabbit lungs and rabbit lungs *in vivo* (albumin passage after 90 min ~12 %) (Verbrugge, Gommers et al. 1996). Although a recent report in which isolated, ventilated and perfused rat lungs were used to measure albumin transport

estimated that approximately 25 % of instilled radio-labeled albumin was cleared from the lung preparations per hour (John, Vogel et al. 2001). One possible explanation for this discrepancy is the different ventilation strategy applied in the two studies. While in our previous trial we used a PEEP of 1 mm Hg (Ghofrani, Kohstall et al. 2001) in the present study we applied a PEEP of 2 mm Hg to prevent any atelectasis, thus it is possible that an enhanced clearance rate was attributable to the increased surface area as consequence of the higher end-expiratory pressure. Importantly and in line with these findings, clearance of radio-labeled sodium from the alveolar space also increased when the PEEP was elevated from 1 to 2 mmHg in the same isolated lung model (Ghofrani, Kohstall et al. 2001; Vadasz, Morty et al. 2005).

However, albumin clearance rates were reported to be markedly different in fluid-filled lungs. Data for albumin clearance in fluid-filled lungs range from 0.007 %/min in rats (Jayr, Garat et al. 1994), 0.02 %/min in rabbits (Hastings, Grady et al. 1992), and 0.06 %/min in sheep (Peterson, Dickerson et al. 1989). Since most work addressing transport properties in the lung was initially done in fluid-filled lungs, investigators initially concluded that the flux of large proteins like albumin through the alveolo-capillary barrier was an extremely slow process and reflected the intactness of the air-blood barrier (Hastings, Grady et al. 1992; Hastings, Wright et al. 1994; Wangensteen, Bartlett et al. 1996). The underlying reason for the apparent discrepancy when compared with the albumin clearance rates in different models is presently unknown. However, a possible explanation might be that the instillate applied in studies where low protein clearance rates were measured generally contained high levels of plasma proteins, (Hastings, Wright et al. 1994; Wangensteen, Bartlett et al. 1996) including native albumin, and it is therefore possible that these investigators characterized the fraction of transalveolar protein transport which was not inhibited by the excess (competing) unlabeled albumin. Thus, these studies probably assessed mainly passive transport of albumin. In contrast, when alveolar instillates contained very low levels of native albumin, a much more rapid transport of labeled albumin occurred (Ghofrani, Kohstall et al. 2001; John, Vogel et al. 2001).

When lungs were maintained at 22 °C or 4 °C, the clearance of albumin from the distal air spaces was markedly decreased (**Figure 8**). This was not due to a change in epithelial permeability since clearance of [^{3}H]mannitol, a marker of passive paracellular movement of solutes and thus epithelial permeability (Effros, Mason et al. 1986), was unaffected by low temperature (**Figure 9**). These findings are in line with several reports suggesting that movement of proteins through the air-blood barrier is temperature-sensitive (Hostetter, Dawson et al. 1981; Serikov, Grady et al. 1993). Movement of albumin at 4 °C is exclusively passive, because all vesicular movement is completely shut down at ~15 °C (Kim and Malik 2003; Hastings, Folkesson et al. 2004) and thus all active transport processes are fully inhibited at 4 °C (Rutschman, Olivera et al. 1993). Since passive transepithelial movement was unaltered under all experimental conditions and ^{125}I-albumin was cleared much faster (approximately 10-fold) at 37 °C than at 4 °C, we concluded that under control conditions (37 °C) the bulk of albumin clearance is probably a result of an active transport process.

Approximately 15 % of ^{125}I was uncoupled from albumin in the alveolar space. Since ^{125}I is a small solute, it can cross the alveolo-capillary barrier via paracellular pathways to a similar extent as sodium (Effros, Mason et al. 1986). In addition to this passive diffusion, it has been well established that ^{125}I, although to a much smaller extent, is transported across the alveolar epithelium through chloride channels, such as the cystic fibrosis transmembrane conductance regulator (CFTR) (Shen, Mrsny et al. 1995). Therefore, clearance of the free ^{125}I might interfere with our measurements accounting for some of the clearance rate we assess for ^{125}I-albumin. However, at 4 °C, when amounts of free ^{125}I were identical to that of measured 37 °C (~15 %; **Figure 10**) the clearance rate of ^{125}I-albumin was measured to be approximately 10 % of the control transport rate at 37 °C. In contrast, passive movement of the small solute [^{3}H]mannitol was found to be unaltered at this temperature. It is important to note that the majority of free ^{125}I movement through the alveolo-capillary barrier is also mediated by passive paracellular transport which remains intact at this temperature. Therefore, although the rate of the radio-labeled albumin clearance

from the alveolar space may be overestimated, it is unlikely that this artifact calls into question the validity of the conclusions we draw from our investigations. Measurements were always made in the presence of this small free ^{125}I fraction and passive paracellular permeability, through which the vast majority of free ^{125}I fraction would leave the alveolar space, was not significantly altered under any experimental conditions. Furthermore, transport of ^{125}I-albumin could be effectively blocked by a variety of interventions, which maintained the level of free ^{125}I fraction in the ELF volume equivalent to that observed in untreated control lungs.

Under control conditions the perfusate of our isolated lung model does not contain any protein. Therefore, an albumin gradient between the alveolar compartment (which contains trace amounts of albumin) and vascular space may exist, which might promote passive movement of the albumin from the distal air space to the vasculature. However, in the presence of excess albumin in the vascular compartment, where this gradient was eliminated no change in the clearance of ^{125}I-albumin from the airspaces occurred. These data further strengthen the hypothesis that albumin clearance from the distal airways is mediated by an active process (**Figure 13**).

Importantly, it was also found that movement of albumin through the alveolo-capillary barrier was vectorial. When FITC-labeled albumin was administered to the vascular compartment of the lung, the transport rate from the vasculature to the alveolar compartment was less dependent on temperature (values were comparable at 37 °C and 4 °C) and were similar to the movement rate measured at 4 °C from the air space to the perfusate. Thus movement of albumin through the air-blood barrier of the lung is facilitated mainly via active transport in a uni-directional manner (**Figure 11**). These data are in line with the recent publication of Kim and coworkers describing a markedly faster albumin transport from the apical to the basolateral, than from the basolateral to the apical direction, in cultured rat alveolar epithelial monolayers (Kim, Matsukawa et al. 2003).

The high albumin clearance rate observed in our isolated ventilated lung preparation was not due to albumin tracer dissociation (**Figure 10**) or fragmentation of the protein (**Figure 12**). Moreover, when the protease activity of the ELF was inhibited by the metallo protease inhibitor EDTA or the serine protease inhibitor AEBSF (Gross 1995; Greenlee, Werb et al. 2007) no effect on ^{125}I-albumin clearance from the alveolar space was observed (**Figure 12**). Since it was found that albumin transport accross the alveolo-capillary barrier was an active, temperature-dependent and uni-directional process that was independent of albumin degradation by proteases in the ELF, it followed that intact albumin was probably taken up by the alveolar epithelium. Therefore, the focus was shifted to the identification of the mechanism by which albumin uptake by the alveolar epithelium occurred, and how subsequent albumin transport across the alveolo-capillary barrier was regulated.

Albumin absorption across the alveolar epithelium could be affected by two pathways: (1) non-specific endocytosis (macropinocytosis) or (2) receptor-mediated endocytosis. However, it is well established that macropinocytosis occurs at a much lower rate than estimated for albumin transport (John, Vogel et al. 2001; Kim and Malik 2003). Furthermore, pinocytic uptake does not saturate with increasing concentrations of exogenous proteins, and is not competed by the presence of other macromolecules, as opposed to the saturable receptor-mediated endocytosis processes (Conner and Schmid 2003). To determine the route through which ^{125}I-albumin was taken up by the alveolar epithelium we measured ^{125}I-albumin clearance from the alveolar airspaces in the presence of a 1000-fold molar excess of unlabeled albumin. Deposition of the excess native albumin into the alveolar space significantly decreased the active transport of ^{125}I-albumin out of the air spaces, however, somewhat surprisingly, only about 30 % of the active albumin transport was inhibited (**Figure 13**). In contrast, when similar experiments were performed in cultured alveolar epithelial cells, a 1000-fold molar excess of unlabeled albumin resulted in an almost complete block of radio-labeled albumin uptake (Figure 14). We believe that the somewhat less marked effects of excess albumin in our intact lung preparation probably resulted from

the inability of our aerosol delivery system to deposit the desired amount of albumin to the alveoli. The aerosolized albumin solution is very viscous and thus it is very difficult to aerosolize. Thus, it is probable that only a fraction of the desired amount of native albumin reached the lungs. In any case, these data suggest that a receptor-mediated, saturable process is involved in the uptake of albumin by the alveolar epithelium.

The notion for an albumin receptor was first proposed in the endothelium, in which a ~60 kDa plasma membrane sialoglycoprotein (gp60) was identified (Schnitzer, Ulmer et al. 1990). This protein was shown to specifically bind to albumin (thereby promoting its uptake by endothelial cells) but not other macromolecules (Schnitzer 1992). In line with our findings, gp60 has recently been described in the alveolar epithelium and identified as an important mediator of albumin uptake in these cells (John, Vogel et al. 2001; Kim and Malik 2003). However, several other receptors for albumin have been identified including the multiligand, endocytic receptors cubilin and megalin (Birn and Christensen 2006). The ~280 kDa cell surface glycoprotein, cubilin was originally isolated as the intrinsic factor/vitamin B12 receptor from the small intestine and was later found to be also highly expressed in the kidney and in epithelial cells of the yolk sac (Moestrup and Kozyraki 2000). Although cubilin does not contain transmembrane sequences it interacts with megalin which contains a short transmembrane sequence to mediate particulate uptake of macromolecules such as high-density lipoproteins (HDL) (Moestrup, Kozyraki et al. 1998). Importantly, cubilin and megalin were recently shown to be essential for albumin reabsorption from the proximal tubule of the kidney (Birn, Fyfe et al. 2000). Cubilin and megalin have been recently reported to be expressed in the lung, where they appear to mediate HDL uptake by the alveolar epithelium (Kolleck, Wissel et al. 2002). Thus, there are several receptors which could bind albumin in the alveolar space of the lung thereby promoting its uptake by the epithelium.

Receptor-mediated endocytosis of macromolecules is carried out by caveolae-mediated and clathrin-dependent pathways. Both caveolin-coated plasmalemmal vesicles (caveolae) and clathrin-coated pits are present in alveolar

type I and type II cells suggesting possible uptake of proteins via these structures (Mostov and Cardone 1995; Kasper, Reimann et al. 1998; Campbell, Hollins et al. 1999; John, Vogel et al. 2001). In the present study, pretreatment of lungs with the caveolae-mediated endocytosis inhibitors filipin or NEM prior to deposition of the radio-labeled albumin into the alveolar compartment resulted in a partial inhibition of ^{125}I-albumin clearance from the distal air space. This was not due to a change in paracellular permeability, since neither of the pharmacological inhibitors had any effect on passive movement of solutes (as assessed by [^{3}H]mannitol flux) through the alveolo-capillary barrier. Unfortunately, it was not possible to directly target the alveolar epithelium with these pharmacological agents since both filipin and NEM alter surface tension thereby preventing aerosolization. For this reason, these inhibitors were applied to the vascular compartment of intact lungs (**Figure 15**). Thus, while it could be concluded that transit of albumin through the alveolo-capillary barrier requires caveolae-mediated endocytosis, it remains uncertain whether this endocytosis occurs in the alveolar endothelium or the epithelium. Indeed, it has been well demonstrated that uptake of albumin by endothelial cells is mediated by caveolae-dependent endocytosis (Drab, Verkade et al. 2001; Schubert, Frank et al. 2001; Vogel, Minshall et al. 2001; John, Vogel et al. 2003; Mehta and Malik 2006). Similarly, it has been recently suggested that inhibition of the caveolae-mediated endocytosis process may result in impaired albumin absorption by the alveolar epithelium (John, Vogel et al. 2001; Kim, Matsukawa et al. 2003). In line with these findings, when filipin and NEM were applied to cultured A549 cells, a similar effect was noted as in the isolated lung setup **(Figure 16)**. Thus, it appears that caveolin function is necessary for the alveolar uptake of albumin and it might also play a role in subsequent transport of the protein through the endothelium.

Clathrin-mediated endocytosis occurs in all mammalian cells and carries out the continuous uptake of nutrients, including several proteins, after binding of the molecules to specific receptors (Conner and Schmid 2003). Coated pits are formed by the assembly of cytosolic coat proteins, the main assembly unit being

clathrin (Owen, Collins et al. 2004). Here, the effect of clathrin inhibitors, chlorpromazine and phenylarsine oxide, were first examined on albumin clearance from the distal air space. Chlorpromazine inhibits the process by causing the loss of coated pits from the cell surface, probably by preventing AP-2 binding to membranes (Wang, Rothberg et al. 1993). Phenylarsine oxide inhibits clathrin-mediated endocytosis by reacting with sulfhydryls to form stable ring structures thereby preventing formation of the clathrin-coated vesicle (Visser, Stevanovic et al. 2004). When either of these inhibitors was deposited into the alveolar space of lungs by aerosolization, a significant decrease in ^{125}I-albumin transport was evident, suggesting an important role for clathrin function in the uptake of albumin by the alveolar epithelium (**Figure 17**). Similarly, when alveolar epithelial cells were treated with these agents albumin uptake was markedly inhibited (**Figure 18**). These observations are in line with a recent finding, which conclusively demonstrated that clathrin-dependent endocytosis was required for the uptake of albumin by cultured RLE-6TN rat alveolar epithelial cells (Yumoto, Nishikawa et al. 2006). However, a role for clathrin-dependent endocytosis in the clearance of proteins from the lung has not yet been reported. In contrast, transport of albumin through the proximal tubule of kidney (Caruso-Neves, Kwon et al. 2005; Birn and Christensen 2006), across the human placenta (Lambot, Lybaert et al. 2006) and through epithelium of the lens (Sabah, Schultz et al. 2007) have been recently described.

Transport of proteins through the epithelium could be mediated by vectorial transport of macromolecules across cells within vesicles, termed transcytosis (Tuma and Hubbard 2003). Alternatively, after internalization proteins might be transported to lysosomes and/or proteasomes for degradation (Rivett 1990). During transcytosis, once the endocytosis is complete, the released vesicles move through the cytoplasm, presumably on prescribed ways, bypassing lysosomes, to the opposite cell surface, where they fuse with the target membrane and release albumin by exocytosis (Mehta and Malik 2006). When the cell-permeable actin stabilizer PO, a potent inhibitor of endo- and transcytosis, was applied into the alveolar space of the intact lungs, a marked block of

^{125}I-albumin clearance occurred (**Figure 20**), confirming the hypothesis that endo- and/or transcytotic events were mediating protein movement across the alveolo-capillary barrier. Since PO alters cytoskeletal function, which is required for both caveolae- and clathrin-mediated endocytosis (Engqvist-Goldstein and Drubin 2003), it inhibits both endocytic pathways. Importantly, administration of PO to lungs resulted in a significantly greater inhibition of albumin transit than did the inhibitors of caveolae or clathrin alone. Furthermore, when A549 cells were treated with the combination of blockers of the caveolin and clathrin function, a strong additive effect of these drugs on albumin uptake was evident (**Figure 19**). These findings strengthen our hypothesis that both endocytic pathways are involved in the transport of albumin.

Since vesicle movement across the cell during transcytosis requires actin, PO is also a potent inhibitor of transcytosis. There are several examples of transcytosis being responsible for transport of proteins across the epithelium and the endothelium. For example, maternal IgG crosses the neonatal rat intestinal epithelium into the blood circulation via specific Fc receptors (Rodewald and Kraehenbuhl 1984), transport of immunoglobulin A from the blood circulation across rat hepatocytes into the bile (Mullock, Jones et al. 1980), and transport of low-density lipoproteins across endothelial cells (Vasile, Simionescu et al. 1983). Transcytosis of albumin has been extensively studied in the endothelium (Tiruppathi, Song et al. 1997; Vogel, Minshall et al. 2001; Minshall and Malik 2006). Although a number of studies have investigated the contribution of transcytosis to clearance of protein from the air spaces, (Kim and Malik 2003; Hastings, Folkesson et al. 2004) its significance in albumin transport remains uncertain.

When lungs were treated with monensin, an inhibitor of cellular vesicular transport, and thus transcytosis, albumin clearance from the lung was significantly inhibited, confirming the notion that transcytosis is required for albumin removal from the alveolar space. However, the degree of inhibition with monensin was not as pronounced as the effect of PO on albumin transport (**Figure 20**). There are two possible explanations to this difference. First, the pre-

incubation time with monensin in our experiments was only 30 min at the time of the start of the radio-labeled albumin deposition and detection. Since clearance measurements were performed over a two-hour time-course the lungs were incubated with monensin for a total of 150 min. In contrast, in some previous reports, a pre-incubation time of two to three hours was employed (Deffebach, Bryan et al. 1996; Sakagami, Byron et al. 2002), although in other studies monensin was applied for rather short times (10 - 60 min) (Fittschen and Henson 1994; Miserey-Lenkei, Parnot et al. 2002). In particular in one report alveolar epithelial cells and rabbit lungs were treated with monensin for one hour prior to measuring protein transport (Hastings, Wright et al. 1994). As discussed in detail in the "Introduction" section, a disadvantage of the isolated, ventilated and perfused lung model is, that longer experiments (more than 4 h) cannot be performed in this setup (Seeger, Walmrath et al. 1994), thus we elected to apply monensin for relatively short times. Therefore it is uncertain whether monensin was able to exhibit its full effect in our intact lung model. Another possible explanation is that while PO blocks both endo- and transcytosis, monensin would primarily inhibit the latter process. For these reasons, it is possible that while the majority of albumin clearance is mediated by an initial endocytosis of albumin by the alveolar epithelium, not the all of the endocytosed albumin is transported intact through the cells. Some of the albumin may undergo modification and subsequent degradation.

In line with this notion, when the intactness of radio-labeled albumin in the vascular space was assessed in control experiments by TCA precipitation or filtering the samples with a 50 kDa cut-off, approximately 50 % of the protein was found to be intact (**Figure 21**). The additional ~50 % could have been resulted from degradation products of ^{125}I-albumin, if the radio-labeled protein was degraded after it was taken up by the epithelium and/or free ^{125}I, which was already unbound in the alveolar space and diffused accross the barrier. When the amount of free ^{125}I was assessed in the perfusate in experiments conducted at 4 °C, almost 90 % of the ^{125}I was found to be unbound in the perfusate. Since paracellular movement of small solutes was unaltered at this temperature (and

under all other experimental conditions), we believe that the amount of free ^{125}I transported at low temperature (~10 %, when compared to controls; **Figure 21**) reflects the contribution of free ^{125}I diffusion to the amount of unbound ^{125}I detected in the vasculature. Taken together, these findings suggest that the majority of unbound ^{125}I detected in the perfusate occurred probably as a consequence of degradation of the radio-labeled albumin either while the protein was crossing the air-blood barrier or in the perfusate. Further research is warranted to address this important issue.

In summary, albumin transport through the alveolo-capillary barrier of intact rabbit lungs is an active, receptor-mediated and saturable process. Excess albumin is taken up by the alveolar epithelium by caveolae- and clathrin-dependent endocytosis and subsequently transported to the vasculature in part intact via transcytosis and in part degraded by a yet unidentified mechanism.

Several important issues remain to be elucidated, which include the identification of specific albumin receptors in alveolar epithelial cells, and the determination of cellular signaling mechanisms involved in alveolar epithelial protein transport. In particular, further studies are warranted to elucidate the regulatory mechanisms underlying alveolar epithelial transport in health and disease. Better understanding of these mechanisms may ultimately lead to novel therapeutic approaches for the treatment of ARDS.

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6. Abstract

A hallmark of acute lung injury/acute respiratory distress syndrome (ALI/ARDS) is the accumulation of protein-rich edema fluid in the distal airspaces, leading to a life-threatening impairment of alveolar gas exchange. Accumulation of serum proteins (such as albumin) in the alveolar space leads to an increased oncotic pressure, thereby preventing fluid reabsorption and resulting in worse outcome. Thus, excess alveolar protein must be cleared for patients with ALI/ARDS to survive. However, the mechanism by which protein is removed from the alveolar space is not fully understood.

To investigate the mechanisms of protein transport across the alveolo-capillary barrier, an isolated, ventilated and perfused rabbit lung model was employed. Additional experiments in human epithelial A549 cells were conducted to further evaluate the role of the alveolar epithelium in the transport of albumin. Radio-labeled albumin (^{125}I-albumin) was deposited into the distal air spaces of isolated, ventilated and perfused rabbit lungs by ultrasonic nebulization, and clearance of ^{125}I-albumin from the alveolar space was measured in real-time by gamma detectors placed around the lungs and around the perfusate reservoir. Under control conditions 29.8 ± 2.2 % of the radio-labeled alveolar albumin was transported (in 120 minutes) to the vascular compartment. Experiments conducted at 4 °C or 22 °C resulted in significantly lower albumin clearance (3.7 ± 0.4 or 16.2 ± 1.1 %, respectively), suggesting that the transport of albumin was facilitated by an active process (no changes in paracellular transport rates were observed).

The transport of albumin across the alveolo-capillary barrier was saturable and uni-directional, since deposition of a 1000-fold molar excess of unlabeled albumin into the alveolar space prior to nebulization of ^{125}I-albumin largely inhibited the transport of ^{125}I-albumin to the vasculature, while administration of unlabeled albumin to the vascular space had no effect on albumin clearance. In line with these findings, administration of a 1000-fold molar excess of unlabeled albumin to A549 cells inhibited uptake of ^{125}I-albumin, suggesting that albumin uptake by the alveolar epithelium was receptor-mediated. When lungs were

treated with the transcytosis inhibitor monensin or the actin stabilizer phalloidin oleate, transit of albumin was significantly inhibited. No degradation of protein occurred in the alveolar space, as assessed by measurements of intact albumin from broncho-alveolar lavage samples; and pre-application of protease inhibitors, which had no effect on albumin clearance. These data collectively suggested that intact ^{125}I-albumin was transcytosed by the alveolar epithelium.

The initial step of transcytosis is either clathrin-dependent or caveolae-mediated. To further dissect the mechanism of albumin uptake by the alveolar epithelium, lungs were pretreated with the clathrin-dependent endocytosis inhibitors chlorpromazine or phenylarsine oxide. Inhibition of clathrin-dependent endocytosis resulted in a significant decrease in the albumin transport rate in intact lungs. Furthermore, administration of these inhibitors to A549 cells also decreased ^{125}I-albumin uptake by these cells, confirming that clathrin-dependent endocytosis was important for albumin clearance from the alveolar compartment.

Interestingly, administration of the caveolae-mediated endocytosis inhibitors, *N*-ethylmaleimide or filipin to the isolated lungs also resulted in a significant decrease in albumin clearance from the distal air spaces. Application of caveolae-mediated endocytosis inhibitors to A549 cells resulted in a similar decrease in ^{125}I-albumin uptake. Moreover, co-administration of the clathrin and caveolae inhibitors had an additive effect, suggesting that both endocytic pathways were required for albumin uptake by the alveolar epithelium.

Our data indicate that clearance of albumin from the distal air spaces is facilitated by active transcytosis across the alveolar epithelium of intact rabbit lungs. Both, clathrin- and caveolae-mediated endocytosis may play a role albumin transport from the alveolar compartment to the vascular space. Further understanding of these mechanisms is of clinical importance, since an inability to clear excess protein from the alveolar space is associated with poor outcome in patients with ALI/ARDS. Interfering with the protein clearance mechanisms at play in the lung may ultimately lead to novel therapeutic approaches for the treatment of this disease.

7. Zusammenfassung

Die Entstehung einer proteinreiche Ödemflüssigkeit in den Alveolen ist eine Hauptkomponente im Rahmen der Erkrankung Acute Lung Injury/Acute Respiratory Distress Syndrome (ALI/ARDS), welche zu einer massiven Einschränkung des Gasaustausches führt. Der onkotische Druck im Alveolus steigt massiv an, hält vermehrt Wasser in den Alveolen zurück und die Situation verschlimmert sich weiter. Die Auflösung des Ödems und die Verminderung der Proteinkonzentration sind also wichtigste Punkte für das Überleben des Patienten, wobei die Mechanismen zur Senkung der Albuminkonzentration in den Alveolen nicht vollständig bekannt sind.

In dieser Arbeit wurden die Prozesse der Albumin-Clearence aus dem alveolaren Raum in das Gefäßsystem unter Zuhilfenahme des Modells der isolierten, ventilierten und perfundierten Kaninchenlunge untersucht. Zusätzliche Experimente in humanen epithelialen A549 Zellen wurden parallel durchgeführt, um die besondere Rolle des alveolaren Epithels beim Albumintransport näher zu beleuchten. Durch Vernebelung wurde ^{125}I-Albumin in die isolierten Lungen eingebracht und der Transport dieses Proteins von der alveolaren auf die vaskulare Seite mittels Gamma-Detektoren in Echtzeit gemessen, wobei diese sowohl um die Lunge (Abnahme der Counts während des 120 min dauernden Versuches) als auch um das Perfusat (Zunahme der Counts) platziert worden sind. In Kontrollexperimenten (37°C) wurden in 120 Minuten 29,8 ± 2,2% des eingebrachten ^{125}I-Albumin aus der Lunge in das Perfusate transportiert. Bei 4°C (3,7 ± 0,4%) oder 22°C (16,2 ± 1,1%) Lungentemperatur verminderte sich diese Transportrate signifikant. Daraus folgerten wir, dass dieser Prozess aktiv stattfand, wobei keine veränderten passiven, parazellularen Vorgänge bei verschiedenen Temperaturen beobachtet wurden.

Dieser Transportprozess war sättigbar und uni-direktional, da in die Alveolen eingebrachtes, 1000fach stärker konzentriertes natives Albumin, den Transport von ^{125}I-Albumin ins Perfusate signifikant verminderte. Dagegen hatte die Administration von nativem Albumin in das Perfusat keine Wirkung auf die

Transportrate. Die Aufnahme von ^{125}I-Albumin in A549 Zellen wurde massiv eingeschränkt, wenn kompetitiv natives Albumin beigegeben wurde, was gleichfalls die Theorie einer rezeptorvermittelten Aufnahme des Proteins stützt.

Die Transportrate von ^{125}I-Albumin in der Lunge verminderte sich signifikant, wenn Transzytose Inhibitoren (Phalloidin oleate oder Monensin) durch Vernebelung eingebracht wurden. Daraus folgte, dass ^{125}I-Albumin wahrscheinlich mittels Transcytose in intaktem Zustand durch das Epithel geschleust wird, da Messungen aus brochoalveolaren Lavagen die Unversehrtheit von ^{125}I-Albumin ergaben und vernebelte Protease Inhibitoren keine Änderungen der Transportrate zufolge hatten.

Ausgangspunkt jeder Transzytose ist entweder Clathrin- oder Caveolae-vermittelte Endozytose. Um deren Beteiligung zu eruieren, vernebelten wir Inhibitoren der Clathrin-vermittelten Endozytose (Chlorpromazine oder Phenylarsine oxide) in die Alveolen, was zu einer signifikanten Verminderung der Transportrate von ^{125}I-Albumin führte. Auch in den Zellen (monolayer) führte die Gabe dieser Substanzen zu deutlich geringeren Aufnahmeraten von Albumin. Administration von Inhibitoren der Caveolae-vermittelten Endozytose (NEM oder Filipin) ins Gefäßsystem führte ebenfalls zu erniedrigten Transportraten vom alveolaren Teil in das Perfusat. Die Verabreichung dieser Inhibitoren zu A549 Zellen war ebenfalls mit einem Rückgang in der Aufnahmerate von ^{125}I-Albumin vergesellschaftet. Darüber hinaus führte die Kombination von Inhibitoren unterschiedlicher Endozytosetypen in A549 Zellen sogar zu noch geringeren Aufnahmeraten von ^{125}I-Albumin in die Zellen, so dass wahrscheinlich beide Typen der Endozytose für den Transport von Albumin durch die alveolo-kapillare Barriere vonnöten sind.

Diese Daten zeigen, dass der Transport von Albumin von der alveolaren Seite in das Gefäßsystem aktiv mittels Transzytose von statten geht. Beide Subtypen der Endozytose sind in diesen Prozess involviert. Ein weiteres Verstehen dieser Vorgänge ist von hoher klinischer Relevanz, da das Unvermögen einer adäquaten Protein-Clearence bei Patienten mit ALI/ARDS eng mit den Überlebenschancen dieser Erkrankung zusammenhängt.

8. List of publications and scientific awards

Original article:

The lectin-like domain of TNF-α impoves alveolar fluid balance in hydrostatic lung injury
Vadasz I, Schermuly RT, Ghofrani HA, **Rummel S**, Mühldorfer I, Schäfer KP, Seeger W, Morty RE, Grimminger F and Weissmann N
Crit Care Med [in revision]

Abstracts:

Clearance of excess alveolar protein is mediated by epithelial transcytosis in intact rabbit lungs
Rummel S, Morty RE, Grimminger F, Seeger W and Vadasz I.
Eur Respir J 2007 [accepted]

TGF-β inhibits alveolar fluid reabsorption: a role in ARDS?
Morty RE, Vadasz I, **Rummel S**, Olschewski A, Althaus M, Peters DM, Herold S, Fronius M, Günther A, Seeger W and Eickelberg O.
Eur Respir J 2007 [accepted]

Mechanismen der alveolaren epithelialen Protein Clearance am Model einer intakten Kaninchenlunge
Rummel S, Morty RE, Seeger W and Vadasz I.
113th Kongress der Deutschen Gesellschaft für Innere Medizin
Wiesbaden, April 14-18, 2007

TGF-β impaires alveolar fluid transport *in vivo* by blocking ENaC function

Morty RE, Vadasz I, Olshewski A, **Rummel S**, Herold S, Günther A, Seeger W and Eickelberg O.

Proc Am Thorac Soc 2006 3:A784

Scientific award:

Young Investigator's Award (best poster in pulmonary medicine)

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10. Erklärung

Ich erkläre, dass ich die vorliegende Dissertation selbständig und ohne erlaubte fremde Hilfe und nur mit den Hilfen angefertigt habe, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

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