Supplementary material

Supplementary methods

The isolated and perfused rabbit lung model

The lungs were perfused with 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 (Ghofrani *et al.*, 2001). Approximately 23 ml $NaHCO_3$ (Nabic8.4 %, Braun, Melsungen, Germany) was added to maintain a pH of 7.35 - 7.37. A positive end expiratory pressure (PEEP) of 2 cm H_2O was applied to prevent alveolar collapse. The isolated perfused lung was placed in a temperature equilibrated housing chamber and freely suspended from a force transducer (Hottinger Baldwin, Germany) to continuously monitor the weight of the lung. All lungs included in the study displayed no signs of haemostasis, oedema, or atelectasis. Weight, left atrial pressure, PEEP and pulmonary artery pressure were recorded continuously. The signals were digitalized by an electromechanical pressure converter (Combitrans, Braun, Melsungen, Germany). All analogue signals were amplified and converted to digital signals using PlotIT 3.1 software (Scientific Programming Enterprises, SPE, USA). Substances were delivered to the alveolar space with an ultrasonic nebulizer (Optineb, NEBU-TEC, Elsenfeld, Germany). Approximately 60% of the aerosol (~1 ml) reached the lung the bulk of which was deposited into the alveolar space (Vadasz *et al.*, 2005a; Vadasz *et al.*, 2005b).

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 *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 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. The amount of γ-emission of the fractions was quantified by a γ-counter (Packard, Dreieich, Germany).

References

Ghofrani HA, Kohstall MG, Weissmann N, Schmehl T, Schermuly RT, Seeger W & Grimminger F. (2001). Alveolar epithelial barrier functions in ventilated perfused rabbit lungs. *Am J Physiol Lung Cell Mol Physiol* **280,** L896-904.

Hastings RH, Folkesson HG, Petersen V, Ciriales R & Matthay MA. (1995). Cellular uptake of albumin from lungs of anesthetized rabbits. *Am J Physiol* **269,** L453-462.

Vadasz I, Morty RE, Kohstall MG, Olschewski A, Grimminger F, Seeger W & Ghofrani HA. (2005a). Oleic acid inhibits alveolar fluid reabsorption: a role in acute respiratory distress syndrome? *Am J Respir Crit Care Med* **171,** 469-479.

Vadasz I, Morty RE, Olschewski A, Konigshoff M, Kohstall MG, Ghofrani HA, Grimminger F & Seeger W. (2005b). Thrombin impairs alveolar fluid clearance by promoting endocytosis of Na+,K+-ATPase. *Am J Respir Cell Mol Biol* **33,** 343-354.

Supplementary figure legends

Supplementary figure 1: Decreased albumin binding and transport is not due to degradation

(A) ^{125}I-albumin in the alveolar space remains intact throughout the experiment

Intactness of ^{125}I-albumin was assessed by trichloracetic acid precipitation before (start), directly after nebulization (after neb), and at the end of experiment (end). Data represent the mean ± SD, n=6. (B) 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 additional experiments, AEBSF (blue) or EDTA (green) was nebulized to lungs, ^{125}I-albumin tracer was deposited into the alveolar space, and elimination of this tracer from the lung was monitored. Each data point represents the mean of six independent experiments.

Supplementary figure 2: Caveolae-mediated endocytosis does not contribute to alveolar epithelial albumin transport

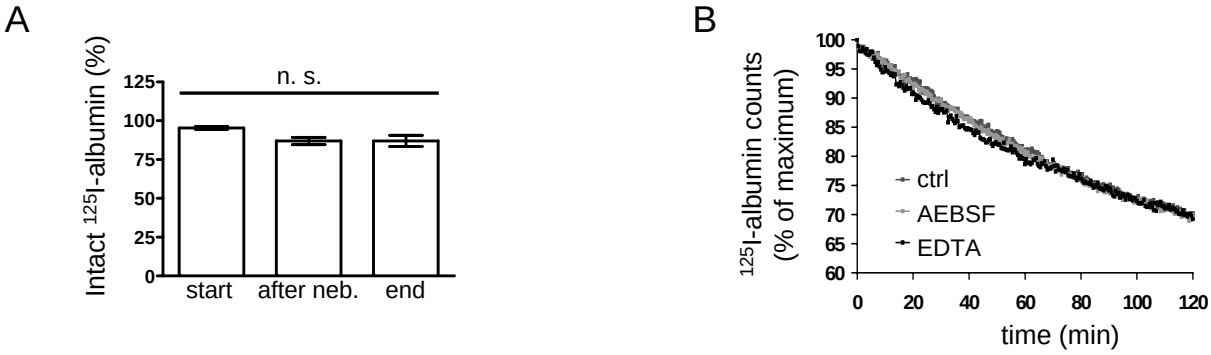
RLE-6TN cells were plated on permeable supports and used for experiments on day 7. Binding (A and B), uptake (C and D) and transport (E and F) of FITC-labelled albumin was assessed by fluorescence. The caveolae inhibitors 100 µg/ml *N*-methylmaleimide (NEM) (A, C and E) und 2 µg/ml filipin (B, D and F) were applied 1 hours prior to incubation with 50 µg/ml FITC-albumin. Binding, uptake and transport of FITC-albumin were measured after 4 hours. n=3; data represent the mean ± SEM.

Supplementary figure 3: RLE-6TN cells have similar properties as AT I like and AT II cells and are therefore a suitable system to study transepithelial albumin transport

RLE-6TN cells were plated on permeable supports and used for experiments on day 7. Binding (A) and uptake (B) of ^{125}I-albumin to RLE-6TN cells is blocked by excess native

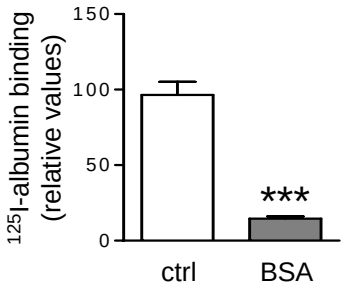
albumin. The amount of surface bound ^{125}I-albumin was assessed by detection of γ-radiation, n=4. (C) Uptake of FITC-albumin by RLE-6TN cells is blocked by excess native albumin. FITC-albumin uptake was assessed by density measurement, n=3. (D) Transepithelial transport of ^{125}I-albumin across monolayers of RLE-6TN cells, n=4; data represent the mean ± SEM **p<0.01, ***p<0.001.

Supplementary figure 1



Supplementary figure 2

A



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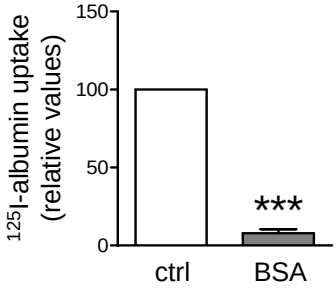

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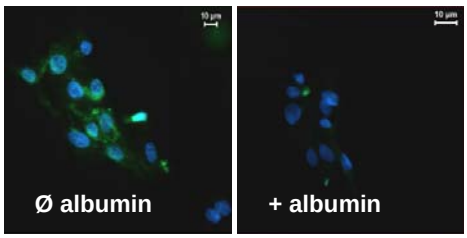

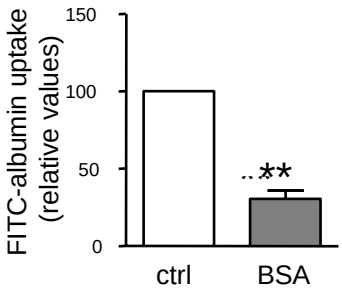

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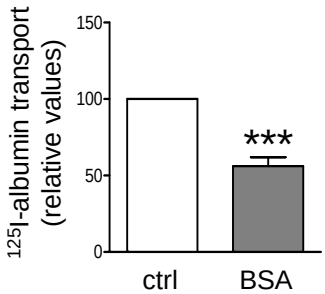

Supplementary figure 3

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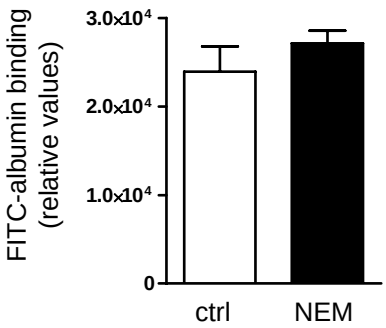

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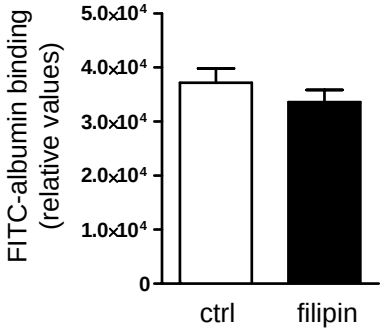

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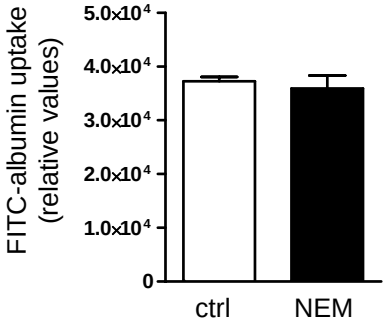

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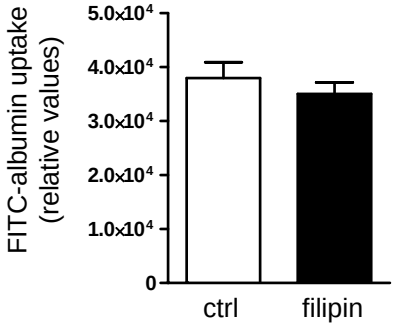

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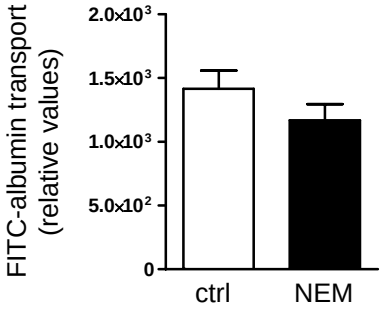

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