Colloidal gold particles as a new in vivo marker of early acute lung injury

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In vivo techniques to quantify and describe the permeability of the lung vasculature are vital not only for the assessment of adequate therapeutic interventions, but also for the development of novel treatment strategies against acute lung injury.

For various clinical situations of systemic inflammation it has been shown that derangement of the endothelial barrier plays a pivotal role in the manifestation of early acute lung injury. We present a novel and sensitive technique that brings microanatomical visualization and quantification of microvascular permeability in line. White New Zealand rabbits were anesthetized and ventilated mechanically. Rabbit serum albumin (RSA) was labeled with colloidal gold particles. We quantified macromolecular leakage of gold-labeled RSA and thickening of the gas exchange distance by electron microscopy, taking into account morphology of microvessels. The control group receiving a saline solution represented a normal gas exchange barrier without extravasation of gold-labeled albumin. Infusion of lipopolysaccharide (LPS) resulted in a significant displacement of gold-labeled albumin into pulmonary cells, the lung interstitium, and even the alveolar space. Correspondingly, intravital fluorescence microscopy and digital image analysis indicated thickening of width of alveolar septa. The findings were accompanied by a deterioration of alveolo-arterial oxygen difference, whereas wet/dry ratio and albumin concentration in the bronchoalveolar lavage fluid failed to detect that early stage of pulmonary edema. Inhibition of the nuclear enzyme poly(ADP-ribose) synthetase by 3-aminobenzamide prevented LPS-induced microvascular injury. To summarize: colloidal gold particles visualized by standard electron microscopy are a new and very sensitive in vivo marker of microvascular permeability in early acute lung injury. This technique enabling detailed microanatomical and quantitative pathophysiological characterization of edema formation can form the basis for evaluating novel treatment strategies against acute lung injury.

lipopolysaccharide; microvascular permeability; electron microscopy; intravital microscopy; poly(ADP-ribose) synthetase

IN 1967 ASHBAUGH ET AL. (2) described acute respiratory failure. For various clinical situations of systemic inflammation it has been shown that derangement of the endothelial barrier plays a pivotal role in the manifestation and progression of acute lung injury (4, 22, 24, 25). Leukocyte/endothelial cell interactions, as well as the release of proinflammatory cytokines following systemic inflammation, result in perfusion failure and endothelial injury (7, 52). Upon activation, leukocytes may contribute to tissue damage, resulting in capillary leakage and edema generation due to release of proteases and of oxygen and nitrogen radicals forming the highly reactive peroxynitrite (6, 15, 54). Nitrated albumin being measurable created from the reaction of native albumin with peroxynitrite is able to open interendothelial junctions (42). Furthermore, peroxynitrite can, among other impairments, activate the nuclear enzyme poly-(ADP-ribose) synthetase (PARS) via DNA damage (49, 50, 56). There is strong evidence that PARS activation is supporting a vicious circle of further inflammatory cell accumulation through upregulation of adhesion molecules on the one hand (19, 20); on the other hand, PARS activation results in a massive and fatal energy depletion of affected endothelial cells.

Injury of the endothelial lining is accompanied by enhancement of microvascular permeability in particular to large molecules, thereby leading to lung edema formation (36, 45). Capillary leakage with fluid loss into the lung interstitium contributes to respiratory failure clinically appearing as respiratory distress and pulmonary infiltrates on chest X-ray films (40). Understanding the very early and microanatomical pathophysiological processes is indispensable for the development of adequate therapeutic interventions.

Current research techniques to quantify and describe the severity of acute lung injury in rodents were recently summarized (38). Among others, gravimetric techniques (5, 12, 39) and measurements of albumin or other proteins in bronchoalveolar (BAL) fluid (51) rank with the standards. Currently, radiotracer methods (9, 29, 34) unambiguously represent the "gold standard" for detecting pulmonary edema not least because they allow a reliable, quantitative, and highly sensitive assessment of the permeability of the lung. Adequate and immediate visualization of microanatomical processes in and around single capillary cells is exclusively possible by means of electron microscopy (3, 31). A highly sensitive tool that brings microanatomical and subcellular alterations in line with the description and quantification of permeability during early acute lung injury has been missing up to now.

The aim of our study was the direct visualization and quantification of microvascular macromolecular permeability of the endothelial barrier in early acute lung injury using a technique with high resolution. Furthermore, we wanted to investigate whether early endothelial damage is associated with a quantitatively assessable capillary leak of macromolecules. Additionally, the mechanisms of protein transport are still controversial (33, 38, 43): is a transcellular active vesicle shuttle or a paracellular protein exchange predominant? Our study was also initiated to further elucidate that subcellular protein traffic.

We therefore modified a technique to detect gold-labeled albumin by standard electron microscopy originally described for examination of microvessels perfusion (21). This new technique is supposed to allow accurate assessment of the displacement of macromolecules across the endothelial barrier and to form the basis for evaluating novel treatment strategies in early acute lung injury.

* K. Heckel and R. Kiefmann contributed equally to this study.

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METHODS

Animals and anesthesia. Male New Zealand White rabbits ranging in weight from 2.5 to 3.3 kg were anesthetized by intravenous application of 50 mg of thiopental sodium followed by 50 mg/kg body weight (bw) α-chloralose. Piritramide (0.5 mg/kg bw) and pancuronium bromide (0.3 mg/kg bw) were administered for analgesia and muscle relaxation. The surgical preparation and experimental setup have previously been described in detail (26). Briefly, the animals were tracheotomized, intubated, and pressure-controlled ventilated [inspiratory oxygen fraction (FiO2), 0.4; inspiratory airway pressure, 8 mmHg; expiratory airway pressure, 2 mmHg; infant ventilator model IV-100; Kontron, Eching, Germany]. Catheters were introduced into the carotid artery and the pulmonary artery for continuous measurement of arterial blood pressure and pulmonary artery pressure (PAP). The fourth and fifth ribs of the right chest were partially removed, and a transparent window was implanted instead. Changes in microvascular permeability were visualized by intravital fluorescence microscopy (25).

After review and approval of the local ethics committee and the government of Bavaria, all animals have been treated humanely in accordance with “principles of laboratory animal care” (NIH Publication No. 86-23, revised 1985) as well as with the German legislation on protection of laboratory animals.

Preparation of rabbit serum album-gold complexes. The production of rabbit serum album (RSA)-gold complexes was performed as described previously (21). Solution A [2,400 ml; 2,370 ml H2O plus 30 ml 1% (wt/vol) HAuCl4 (Fluka, Buchs, Switzerland) in H2O] was heated to 60°C and quickly mixed with solution B [450 ml H2O, 120 ml 1% trisodium citrate, and 6 ml 1% tannic acid (Mallinckrodt, Paris, KY) also heated to 60°C]. In a first concentration step, the gold colloid was gently boiled down to ~25% of its original volume (concentrated gold solution). To adjust the pH, 25 ml of a 0.2 M sodium phosphate buffer (pH 6.1) were added to 500 ml of colloidal gold with rapid stirring. A 5% (wt/vol) solution of RSA in H2O was prepared, and serial dilutions of this were used to determine the minimal amount of RSA that would stabilize the gold against electrolyte-induced aggregation. This was 0.0643 mg of RSA per milliliter of concentrated gold solution. The crude complex was then prepared by rapidly mixing 525 ml of concentrated gold solution (pH 6.1) with 0.675 ml of 5% RSA in 10 mM sodium phosphate buffer. For further concentration, batches of the RSA-gold suspension were centrifuged for 90 min at 35,000 g. The supernatant was discarded, and in a further step at the same centrifugation speed and a running time of 150 min the final concentration to 4–5 ml was performed. After dialysis against Ringer solution (8.0 g NaCl, 0.2 g CaCl2·6 H2O, 0.1 g KCl, and 0.1 g NaHCO3 per 1,000 ml H2O, pH 7.4), the concentrate was frozen drop by drop into liquid nitrogen, and thereafter it was stored at −80°C. Before use, the gold solution was warmed to body temperature and passed through 0.25-μm filters (Millipore, Billerica, MA; previously flushed with Ringer solution). An aliquot of 2 ml/kg bw gold concentrate was intravenously injected within a 15-s period 10 min before termination of experiments.

Determination of gold concentration by spectrophotometry. To find the half-life of gold-labeled albumin in vivo, eight animals were anesthetized and treated as described. Thirty minutes before termination of the experiment, 2 ml/kg bw of the gold solution was administered intravenously. Plasma samples were taken before and 2, 15, and 30 min after injection. The concentration of gold in plasma was determined spectrophotometrically (DU 7500 Spectrophotometer; Beckmann Instruments, Fullerton, CA) by measuring the optical density at 525 nm wavelength (OD525) (18). Plasma gold concentration was measured against plasma samples taken before gold injection.

Determination of extravasation of gold-labeled albumin by electron microscopy. The extravasation of intravenously applied gold-labeled RSA was investigated by electron microscopy to determine pulmonary capillary leakage. After circulation was stopped (10 min after gold concentrate application), the rabbit’s chest was opened, and the lung was carefully removed. Visible surgical alterations of the lung or of parts of it resulted in exclusion from further investigation. The lung was fixed by instillation of 1% glutaraldehyde solution (in 0.1 M Na-cacodylate buffer, pH 7.4, 300 mosM added with 3% dextran) through the tracheal cannula (pressure 25 cmH2O). Five biopsies per rabbit were taken from two subpleural and three central areas of the lung. After postfixation in 2% OsO4, the biopsies were treated according to standard electron-microscopic techniques to dehydrate and embed the tissue in araldite. Sections were cut at 60 nm and mounted on uncoted 200-mesh grids after being stained with uranyl acetate and lead citrate. For investigation of the sections, a Philips CM 10 electron microscope was used. Five sections from each of the five biopsies were analyzed per rabbit lung. The description of the microanatomy followed the criteria of Bachofen and Weibel (4). The location of gold particles was noted, and the length of the gas exchange distance was measured as the mean of at least five closest distances (per section) between the vascular membrane of the endothelium and the alveolar membrane of epithelial cells.

Quantification and distribution of vesicles and gold particles. For quantification of vesicles and gold particles, five electron microscopy photographs per rabbit from different portions of its lung were digitized and analyzed by means of a digital image-processing system (Optimas; Bioscan, Edmonds, WA). The areas of the plasma compartment, the endothelium, and epithelium were measured. Assuming a thickness of 60 nm per section, we calculated the concentration of vesicles and gold particles in each compartment. Per rabbit >1,000 gold particles were counted, and their distribution among plasma compartment, vesicles, interstitium, and alveolar space was notified.

Determination of edema formation by intravital fluorescence microscopy. Subpleural microvessels and alveoli were labeled with rhodamine 6G (0.3 ml/kg bw of a 0.2-mmol saline solution; Merck, Darmstadt, Germany) and visualized by a fluorescence microscope (Leica, Wetzlar, Germany) during prolonged inspiration periods of 10 s. Video recordings were made by a silicon-intensified video camera (C2400–08; Hamamatsu, Herrsching, Germany) on an S-VHS video recorder (AG-7350; Panasonic, Munich, Germany). Edema formation was quantified offline using a digital image-processing system (Optimas, Bioscan) as described previously (25). It was assessed from the width of alveolar septa determined as the mean of at least 10 closest distances between the inner walls of adjacent alveoli. For baseline and repeated measurements the same alveoli per experiment were measured.

Determination of edema formation by lung wet/dry ratio. To assess pulmonary edema formation, lung tissue wet/dry weight ratio was determined by drying samples at 100°C for 24 h.

Albumin concentration of the BAL fluid. After termination of the experiment, the upper lobe bronchus of the rabbit lung was cannulated. The lobe was rinsed with a total of 30 ml of phosphate-buffered saline (PBS). The albumin concentration of the samples was quantified by standard clinical chemistry technique.

Determination of peroxynitrite. Because peroxynitrite seems to be a key player in acute lung injury, peroxynitrite formation was determined spectrophotometrically by quantifying the concentration of thiobarbituric acid-reactive substances (TBARS) in plasma as described previously (13, 30, 37). Fifty microliters of 50% (wt/wt) trichloroacetic acid and 75 μl of 1.5% thiobarbituric acid (Sigma, Deisenhofen, Germany) in 0.3% (wt/vol) NaOH were added to 200 μl of plasma samples. After incubation at 90°C for 60 min and subsequent cooling in ice water, the samples were centrifuged. Finally, 200 μl of samples were transferred to a microplate, and absorbance at 530 nm minus absorbance at 630 nm was read in a microplate reader (Nunclon Delta, Roskilde, Denmark). TBARS were quantified by a standard curve of malondialdehyde (Dynex Technologies, Denkendorf, Germany).
the lung against LPS, the animals (experiments in which we showed that a slight pulmonary edema is
3-aminobenzamide (3-AB, Sigma; 10 mg/kg bw as bolus and 2.5
continuously. Ten minutes before the end, concentrated gold solution
was repeated every 30 min. Macrohemodynamics were registered
according to the formula

\[
\frac{\text{OD525}}{\text{H11005}} = \frac{\text{H11021}}{\text{H11005}} - \text{H11005}
\]

Macrohemodynamics, mmHg

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>2 h</th>
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<tr>
<td>MAP</td>
<td>control</td>
<td>87.8±4.3</td>
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<tr>
<td></td>
<td>LPS</td>
<td>85.0±2.9</td>
</tr>
<tr>
<td></td>
<td>LPS + 3-AB</td>
<td>81.3±4.2</td>
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<tr>
<td>PAP</td>
<td>control</td>
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<td></td>
<td>LPS</td>
<td>15.1±0.72</td>
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<tr>
<td></td>
<td>LPS + 3-AB</td>
<td>15.3±1.40</td>
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Values are means ± SE. Control group (n = 5), LPS group (n = 8), LPS + 3-AB group (n = 7). *P < 0.05 vs. baseline, Wilcoxon signed-rank test. **P < 0.05 vs. control, ANOVA on ranks and Tukey. With respect to the exclusion criteria, all animals were macrohemodynamically stable. MAP, mean arterial blood pressure; PAP, mean pulmonary arterial blood pressure; 3-AB, 3-aminobenzamide.

For additional investigation of peroxynitrite production we detec-
ted nitrotyrosine qualitatively by immunohistochemistry. An im-
portant reaction of peroxynitrite in vivo is the nitration of tyrosine

c. Binding of the primary antibody was detected
through avidin-biotin peroxidase complex formation with a biotin-
conjugated goat anti-mouse IgG (ABC-Kit; Dianova, Hamburg, Ger-
many). The alveolo-arterial oxygen difference (\(A-aD_{\text{O}_2}\)) was calcu-
lated according to the formula

\[
\text{\(A-aD_{\text{O}_2}\)} = [\text{\(\text{P}_{\text{O}_2}\)} - \text{\(\text{Fi}_{\text{O}_2}\)} - \text{\(\text{Pa}_{\text{CO}_2}\)} - 0.8] - \text{\(\text{Pa}_{\text{CO}_2}\)}
\]

Experimental protocol. After surgical preparation and confirming
of the exclusion criteria as mean arterial pressure <60 mmHg, lack of
macroscopic visible atelectasis, hemorrhage, or perfusion failure on
the lung surface, we randomly divided the animals into three groups.
The animals of the control group (n = 5) received a saline solution
(0.9% NaCl; 2 ml/kg bw as bolus and 2 ml·kg\(^{-1}\)·h\(^{-1}\) per infusion).
Systemic inflammation was induced by intravenous application of
endothoxin (LPS from Escherichia coli 0111:B4, 100 µg as bolus and 20 µg·kg\(^{-1}\)·h\(^{-1}\) per infusion; Sigma) to the animals of the LPS
group (n = 8). That dose of LPS was chosen according to previous
experiments in which we showed that a slight pulmonary edema is
induced by this procedure (22). For an intervention that could protect
the lung against LPS, the animals (n = 7) of the third group received
3-aminobenzamide (3-AB, Sigma; 10 mg/kg bw as bolus and 2.5
mg·kg\(^{-1}\)·h\(^{-1}\) per infusion) in addition to LPS (LPS + 3-AB group).
3-AB is a well-known competitive inhibitor of the nuclear enzyme
PARS (47, 48) that seems to play a pivotal role in the pathogenesis of
acute lung injury (19, 20).

Intravitral microscopy was performed under baseline conditions and
was repeated every 30 min. Macrohemodynamics were registered
continuously. Ten minutes before the end, concentrated gold solution
was applied. After killing the rabbit, we immediately opened the chest
and prepared the lungs for electron microscopy.

Statistics. Data are presented as means ± SE. Comparisons be-
tween the groups were tested by ANOVA on ranks and Dunn’s or
Tukey’s. Repeated measurements were tested by Wilcoxon’s signed-
rank test. Statistical significance was given when P < 0.05. Calcula-
tions were performed with the computer program SigmaStat (Jandel,
Erkrath, Germany).

RESULTS

Macrohemodynamics. Table 1 summarizes the results of the mean arterial blood pressure (MAP) and the mean PAP at baseline and 2 h after the beginning of the application of NaCl, LPS, and LPS + 3-AB. Under baseline conditions there were no differences between the groups. After 2 h of LPS infusion, MAP was decreased compared with baseline in the LPS group and the LPS + 3-AB group and was decreased compared with control in the LPS + 3-AB group. There was no significant effect of LPS infusion on PAP. With respect to the exclusion criteria all animals were macrohemodynamically stable.

Gold concentration in plasma. The plasma kinetics of gold-
labelled albumin was investigated on the basis of each four
rabbits of the control group and the LPS group. Therefore, 2
ml/kg bw warmed concentrated gold solution was applied intravenously 30 min before termination of the experiment. Before and 2, 15, and 30 min after the injection, plasma samples were taken. Figure 1 shows the concentration of gold in plasma that was determined spectrophotometrically by mea-
suring OD525. Plasma gold concentration was measured against plasma samples taken before gold injection. Within 30 min after the application of gold solution, a continuous decrease of OD525 in the plasma of both groups with a half-life of ~20 min was measured. There was no significant difference between the groups. To detect where gold-albumin extravasates first, the
time of the application was determined as 10 min before the end of the experiment.

Colloidal gold particles in electron microscopy. Colloidal
gold particles appeared as black 8-nm spheres. They were homogenously dispersed in the whole lumen of pulmonary capillaries. The concentration was high enough to recognize gold particles even in capillaries partly filled with blood cells.
The great majority (>95%) of the gold-labeled albumin showed up as single particles with <5% grouped in small clusters (Fig. 2).

Lung microanatomy. All sections of the control group demonstrated normal microanatomy. The gas exchange barrier is extremely thin where epithelial and endothelial basal laminas become fused. The resulting air-blood barrier had a mean thickness of 0.24 μm but measures <0.1 μm at its thinnest parts (Fig. 3A). Under control conditions the basal laminas were intact. The epithelial and endothelial cells fulfilled all criteria of normal anatomy: a vast capillary network covered by thin endothelial cells is surrounded by the alveolar space. Approximately 95% of the alveolar surface is covered by type I alveolar cells, with their multiple cytoplasmatic thin extensions, and ~5% by type II epithelial cells, which are rich in cytoplasmatic organelles as an indication of their active metabolism. In the control group all observed interendothelial cell junctions were closed for gold-labeled albumin.

The essential alteration of the animals who had received LPS for 2 h was a cellular pulmonary edema due to vascular leakage (Fig. 3B). Endothelial and epithelial cells were swollen and richly loaded with vesicles, indicating a boosted endo- and transcytosis activity (Fig. 3, C and D). The basal laminas were still generally intact, but the lung interstitial space was impressively thickened. The interendothelial cell junctions showed partly normal microanatomy but were frequently also open for passive paracellular migration of macromolecules (Fig. 3, E and F). Quantitatively the vast majority (~90%) of albumin extravasation was due to active transcellular transport.

In contrast, additional PARS inhibition in the LPS + 3-AB group resulted in a nearly complete protection of the lung microanatomy against pathophysiological changes (Fig. 4).

Quantification and distribution of vesicles and gold particles. For quantification of vesicles and gold particles five electron microscopy photographs per rabbit were analyzed. LPS infusion resulted in a twofold increase of the concentration of vesicles in endothelial and epithelial cells (Fig. 5). Even clearer was the boost of vesicles storing gold-labeled albumin after LPS (Fig. 6). The additional application of 3-AB almost normalized the endothelial and epithelial transport of vesicles. We counted 5,315 gold particles in the control group (five rabbits), 8,350 particles in the LPS group (eight rabbits), and 5,825 particles in the LPS + 3-AB group (seven rabbits), and their distribution was noted. The concentration of gold particles in the plasma was 2,655 ± 374/femtoliter (fl) in the control group with no difference from the LPS group (2,659 ± 626/fl) and the LPS + 3-AB group (2,688 ± 402/fl) (Fig. 7). Under control conditions the applied gold-labeled albumin was found almost exclusively in the plasma compartment with a minority of extravascular particles. The infusion of LPS over 2 h resulted in a dramatic increase of the concentration of gold particles in the endothelial (fourfold) and epithelial (sevenfold) compartment and could partly be prevented by additional inhibition of PARS by 3-AB. The majority of all counted gold particles in the control group was in the plasma. Only 7% were stored by vesicles with <1% in the interstitial or alveolar space (Fig. 8). In contrast to LPS + 3-AB, LPS application alone was followed by a significant extravasation of gold-labeled albumin into many vesicles, into the interstitium, and even into alveoli (Fig. 9).

Gas exchange barrier. The resulting air-blood barrier composed of the epithelium, the capillary endothelium, and the interstitial space at its thinnest parts was measured as the mean of five closest distances on each of five representative sections per animal. Table 2 shows that the gas exchange barrier was significantly thicker 2 h after LPS infusion compared with control conditions and with the LPS + 3-AB group.

Width of alveolar septa measured by intravital microscopy. At baseline, there was no significant difference between the widths of alveolar septa of the three groups (Fig. 10). According to the extravasation of gold-labeled albumin and the thickened gas exchange distance, the width of alveolar septa increased in the LPS group, indicating onset of edema formation (Figs. 10 and 11). The increase of the width of alveolar septa following LPS infusion was treated with supplementary PARS inhibition.

Determination of edema formation by lung wet/dry ratio. Two hours after the onset of LPS infusion, the wet/dry ratio (3.8 ± 0.1) did not statistically differ from control (4.2 ± 0.2) or intervention groups (3.9 ± 0.15).

Albumin concentration of the BAL fluid. After termination of the experiment, BAL of the left upper lung lobe was performed with a total of 30 ml of PBS. The recovery was 20.6 ± 0.86 ml in the control group and 21.1 ± 0.74 ml in the LPS group with no significant difference between the groups. The concentration of albumin in the BAL fluid was 75 ± 37 mg/l in the control group and 58 ± 33 mg/l in the LPS group with no significant difference between the groups.

Determination of peroxynitrite. Peroxynitrite formation was determined indirectly by quantifying the concentration of TBARS in plasma (Fig. 12) and qualitatively by immunohistochemistry (Fig. 13). Although the measurement of TBARS in plasma did not show any significant differences and changes between the groups, we found clear signs of increased peroxynitrite production following LPS application by means of immunohistochemistry. Unlike in the control group and the LPS + 3-AB group, we found a clear staining of vessel walls and alveolar septa in the LPS group.
Gas exchange. The pulmonary diffusion quality was investigated by means of arterial blood gas analysis. Therefore, $\lambda$-aDO$_2$ was calculated (Table 3). Under baseline there were no differences between the groups, and over a period of 2 h there was no significant change recognized in the control group and the LPS + 3-AB group. In contrast, the LPS group showed a significantly elevated $\lambda$-aDO$_2$ vs. baseline at the time of 2 h as a sign of reduced oxygen diffusion capacity.

DISCUSSION

Acute respiratory failure is a very severe and frequently seen complication after aspiration, polytrauma, burn, ischemia/...
reperfusion, or sepsis. The understanding of the very early pathophysiology in line with microanatomical changes is essential for the development of adequate therapeutic interventions.

The aim of this study was to establish a new and sensitive technique for the determination, direct visualization, and quantification of microvascular macromolecular permeability in early acute lung injury in vivo.

There are numerous methods for the evaluation of transendothelial transport. In vitro, the transport through vascular endothelium can be quantified (1). Assays for the measurement of blood-tissue exchange include the determination of microvascular permeability in isolated perfused organs or isolated microvessels (8). Isolated perfused lung preparations have been a standard tool for assessment of lung vascular injury for more than four decades (38). Mechanisms of edema formation can be reliably separated under defined filtration conditions (11). Transvascular transport of labeled proteins, such as isotope-labeled albumin, in isolated lungs is still a method to determine vascular permeability (44).

Early steps to determine macromolecular leakage in vivo were measurements of macromolecules labeled with dyes or radioactivity in single microvessels at the microscopical level (28). Increased permeability of albumin indicates vascular barrier dysfunction in vivo (10, 45, 55). Transmicrovascular transport of fluorescein isothiocyanate (FITC)-labeled albumin was used to localize protein leakage during lung edema formation by confocal laser microscopy (41). Other authors have described the use of intravital microscopic techniques to visualize extravasation of FITC-stained albumin or rhodamine B (14, 35). These approaches are attractive because they enable analysis of extravasation quantitatively. Intravital microscopy also permits the determination of lung edema formation by measuring width of alveolar septa (25).

![Fig. 4. Electron microscopic photograph of the gas exchange barrier of the LPS + 3-aminobenzamide (3-AB) group at 2 h. Poly(ADP-ribose) synthetase (PARS) inhibition with 3-AB resulted in a nearly complete protection of the lung microanatomy against pathophysiological changes. Arrowhead, gold particle.](image)

![Fig. 5. Concentration of vesicles in endothelial and epithelial cells [vesicles/μl] at 2 h; means ± SE. Control group n = 5, LPS group n = 8, LPS + 3-AB group n = 7. §P < 0.05 vs. control, ANOVA on ranks and Tukey. LPS infusion resulted in a 2-fold increase of the concentration of vesicles in endothelial and epithelial cells. This increase was prevented by additional application of 3-AB for PARS inhibition.](image)

![Fig. 6. Concentration of vesicles storing gold-labeled albumin in endothelial and epithelial cells [vesicles/μl] at 2 h; means ± SE. Control group n = 5, LPS group n = 8, LPS + 3-AB group n = 7. §P < 0.05 vs. control, ANOVA on ranks and Dunn’s. The concentration of vesicles storing gold-labeled albumin was boosted by LPS infusion. That increase was minimized by additional PARS inhibition.](image)

![Fig. 7. Concentration of gold-labeled albumin particles in the plasma compartment, in endothelial and epithelial cells [particles/μl] at 2 h; means ± SE. Control group n = 5, LPS group n = 8, LPS + 3-AB group n = 7. §P < 0.05 vs. control, ANOVA on ranks and Dunn’s. The infusion of LPS over 2 h resulted in a dramatic increase of the concentration of gold particles in the endothelial (4-fold) and epithelial (7-fold) compartment and could partly be prevented by additional inhibition of PARS by 3-AB.](image)
Current research techniques to quantify and describe the severity of acute lung injury in rodents have recently been summarized (38). Among the measurement of the dynamic and static lung compliance (46) and noninvasive imaging techniques (X-ray, computerized tomography, magnetic resonance imaging, positron emission tomography), BAL has been used extensively to detect lung injury, since any increase of plasma extravasation of vascular permeability on an ultrastructural level is still missing. In vitro assays or approaches with isolated perfused organs are compromised by the fact that the preparations are missing. In vitro assays or approaches with isolated perfused organs are compromised by the fact that the preparations are removed from their normal environment and from the influence of physiological controls such as blood flow, intravascular pressure, extracellular fluid volume, or the extent of lymphatic recirculation. Therefore, to obtain detailed knowledge on the mechanisms of transvascular transport, tissues and organs have to be studied in vivo. The application of fluorescent dyes for albumin labeling might compromise intravital microscopic analysis of the microcirculation because fluorescent staining of erythrocytes and leukocytes is indispensable (23, 24, 27, 32). For intravital investigation of the lung surface, epi-illumination microscopy is needed (27). Compared with transillumination microscopy of, e.g., the hamster cheek pouch, the quantification of albumin extravasation is difficult. Additionally, epillumination microscopy is restricted to surface vessels, and its finding cannot be extrapolated to the three-dimensional structure of the lung. Intravital microscopy is supplementarily affected by potential phototoxic properties of the fluorescent dyes and the influence of the surgical trauma for preparation.

### Table 2. Gas exchange barrier

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<th>Control</th>
<th>LPS</th>
<th>LPS + 3-AB</th>
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<tr>
<td>2 h</td>
<td>0.24±0.019</td>
<td>0.44±0.030 **†</td>
<td>0.27±0.032</td>
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</table>

Values are means ± SE. Control group n = 5, LPS group n = 8, LPS + 3-AB group n = 7. **P < 0.05 vs. control, ANOVA on ranks and Dunn’s. The gas exchange barrier (µm) was significantly thicker 2 h after LPS infusion compared with control and LPS + 3-AB groups.

lung weight. To estimate the blood weight, the authors used radioactively labeled red blood cells. Additionally they could quantify the uptake of albumin into the lung tissue using radioactively labeled albumin.

During the last decades, techniques working with radio-tracer-labeled macromolecules (9, 29, 34) to estimate vascular permeability of the lung became established as the gold standard, not least because they allow a reliable, quantitative, and sensitive measurement of vascular permeability (38). However, because all these techniques have inherent disadvantages, a highly sensitive method for description and quantification of permeability on an ultrastructural level is still missing. In vitro assays or approaches with isolated perfused organs are compromised by the fact that the preparations are removed from their normal environment and from the influence of physiological controls such as blood flow, intravascular pressure, extracellular fluid volume, or the extent of lymphatic recirculation. Therefore, to obtain detailed knowledge on the mechanisms of transvascular transport, tissues and organs have to be studied in vivo. The application of fluorescent dyes for albumin labeling might compromise intravital microscopic analysis of the microcirculation because fluorescent staining of erythrocytes and leukocytes is indispensable (23, 24, 27, 32).

For intravital investigation of the lung surface, epi-illumination microscopy is needed (27). Compared with transillumination microscopy of, e.g., the hamster cheek pouch, the quantification of albumin extravasation is difficult. Additionally, epillumination microscopy is restricted to surface vessels, and its finding cannot be extrapolated to the three-dimensional structure of the lung. Intravital microscopy is supplementarily affected by potential phototoxic properties of the fluorescent dyes and the influence of the surgical trauma for preparation.
Intravital and conservative microscopy is limited in resolution. Statements about subcellular processes and early displacement of single macromolecules are impossible. The limitations of the measurement of albumin concentration in BAL and lung tissue wet/dry weight ratio were shown by the results of this study. Neither detected any difference between control and LPS groups, whereas intravital and electron microscopy showed significant distinctions. Hence, wet/dry ratio and albumin concentration in BAL might quantify severe lung injury but are not sensitive enough to track slight, early changes of acute lung injury without taking into account the fact that regional or even microscopical resolution is missing. The widely applied techniques using radioactivated macromolecules for assessment of vascular permeability are not suitable to portray microanatomical and subcellular alterations. The adequate and immediate visualization of microanatomical processes in single capillary cells is exclusively possible by means of electron microscopy (3, 31).

A highly sensitive tool that brings microanatomical and subcellular alterations in line with the description and quantification of permeability during early acute lung injury has been missing up to now.

We established a new technique to visualize extravasation of albumin by standard electron microscopy to determine lung edema formation. Therefore, we used the procedure described previously to obtain RSA-gold complexes with a defined diameter of 8 nm (21). Using that new albumin marker, these authors (21) investigated plasma perfusion of different organs by electron microscopy under physiological conditions. The production of complexes between gold particles and biological relevant macromolecules is well established (16–18, 41, 53).

The advantage of gold-stained albumin visualized by electron microscopy is that the route of albumin during lung capillary leakage can be detected at the highest possible resolution, avoiding fluorescent and radioactive dyes. Any region of the lung can be analyzed, which makes the results representative for the whole organ. The choice of marker was dictated by the fact that capillaries are unambiguously identifiable only by electron microscopy (3, 31). We therefore looked for a marker that can be clearly identified by standard electron microscopy. The plasma concentration of the marker must be high and measurable without inducing any side effects. Colloidal gold particles fulfill all these requirements. By injection of an aliquot of 2 ml/kg bw gold solution, the concentration of
Although electron microscopy allows both the identification of gold-labeled albumin on its transendothelial pathway as well as the description of microanatomy, there are some disadvantages accompanied with that technique. According to other microscopic methods, the parts of the organ that can be analyzed by electron microscopy are small. Furthermore, a continuous observation of the microvascular network is impossible. A certain number of snapshots represent pathophysiological processes.

Five biopsies per rabbit were taken from two subpleural and three central areas of the lung with no difference between the groups concerning the collecting areas of the biopsies. The microanatomical results also did not show any differences regarding the collecting areas. Therefore, we conclude that the systemic LPS administration created a relatively homogenous lung injury, and sampling issues were not relevant in our study. But the methods for tissue sampling are of course important with more heterogeneous forms of lung injury, like intralveolar-applied noxious substances. We think that our new technique is able to describe and to quantify a more heterogeneous injury, with the one reservation that the tissue sampling needs to be prospectively and exactly regulated or blinded with a probably higher number of samples distributed across the lung.

A subject for discussion is the influence of gold on the extravasation of albumin. Currently it is not clear whether gold-stained albumin compared with native albumin extravasates more slowly because of its increased weight or even more quickly because gold staining could be a stimulus for endocytosis. In agreement with previous results, we found a continuous decrease of the gold concentration in plasma measured by means of optical density (OD525) with a half-life of ~20 min (21). In the lungs of the control group, we found the majority (>90%) of applied gold-labeled albumin in the plasma compartment with ~7% incorporated in vesicles as a sign for active transcytosis. Endo- and transcytosis are well known for native and for labeled albumin (42). Using mouse lungs perfused in situ with a gold-stained albumin solution, Predescu et al. (42) showed that, as early as 30 s after the beginning of perfusion, gold particles were bound to the luminal endothelial plasma-lemma and to open caveolae. Consecutively, they were then actively transported inside of caveolar cavities. Endocytosis of particles by endothelial cells in liver sinusoids is also described as early as 2 min after injection, and preliminary experiments of König et al. (21) have shown that larger particles are even more rapidly eliminated from the blood by increased endocytosis into the reticuloendothelial system. In agreement with recent data (42), almost no interstitial gold particles could be found in the control group, supporting our findings that the interendothelial junctions were intact.

Table 3. 

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LPS</th>
<th>LPS + 3-AB</th>
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</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>108.8±5.4</td>
<td>99.0±6.1</td>
<td>109.0±3.9</td>
</tr>
<tr>
<td>2 h</td>
<td>111.0±6.4</td>
<td>117.1±9.9</td>
<td>113.0±8.7</td>
</tr>
</tbody>
</table>

Values are means ± SE. Control group n = 5, LPS group n = 8, LPS + 3-AB group n = 7, *P < 0.05 vs. baseline, Wilcoxon signed-rank test. Unlike the control group and the LPS + 3-AB group, alveolo-arterial oxygen difference (ΔaDO2 [mmHg]) was significantly elevated 2 h after LPS infusion as a sign of reduced oxygen diffusion capacity.
Bachofen and Weibel (4) described micromorphological alterations in lung tissue of nine patients with sepsis-induced lung injury. In the initial stage, the essential alteration was a cellular pulmonary edema due to vascular leakage. Although the alveolar septa were thickened by migrated cells and interstitial edema, their gross architecture seemed fairly well preserved. Endothelial changes in the acute stage of lung injury were a cytoplasmic cellular swelling and an irregular thickening by large vacuoles. At the epithelial side, areas of normal appearance were suddenly interrupted by areas of total destruction, with complete denudation of the basement membrane. The alterations of the lung ultrastructure we found 2 h after induction of systemic inflammation were strictly in accordance with the findings of Bachofen and Weibel (4).

The infusion of LPS multiplied the endothelial and epithelial concentration of vesicles and gold-stained albumin compared with control. Transendothelial transport of gold particles in vesicles as a sign for active transcytosis was boosted approximately fivefold. Additionally, a significant amount of gold accumulated in the interstitium (13.6%) and even in the alveoli (10.9%), suggesting a second path for extravasation.

Knowing that nitrated albumin, being measurably created under inflammatory conditions from the reaction of native albumin with the highly reactive substrate peroxynitrite, is able to open interendothelial junctions (42), we believe that nitrated albumin might open the second path for extravasation: macromolecules streaming passively through open interendothelial junctions.

For quantification of peroxynitrite production we used a common indirect assay that measures the concentration of TBARS in plasma. TBARS are produced predominantly by lipid peroxidation, the reaction of the highly aggressive peroxynitrite with cellular lipids (13). Application of LPS for 2 h ended up in a strong tendency for increased peroxynitrite production.

For additional investigation of peroxynitrite production, we detected nitrotyrosine qualitatively by immunohistochemistry of paraffin-embedded lung sections. An important reaction of peroxynitrite in vivo is the nitration of tyrosine molecules forming nitrotyrosine-containing proteins (6) that can be detected with a monoclonal antinitrotyrosine antibody. Unlike in the control group, in the LPS group we found a clear staining of vessel walls and alveolar septa, indicating an elevated peroxynitrite production. These results support our hypothesis that peroxynitrite is generated during systemic inflammation, induces the production of nitrated albumin, and is therefore responsible for opening interendothelial junctions.

The quantitative relevance of paracellular flux of proteins is still a subject of discussion (38). The description of vesicles in endothelial cells, the rapid uptake of labeled proteins and their appearance on the abluminal side of the endothelium have been used to emphasize the concept of an active shuttle of proteins by vesicles (33). Other authors (43) support a paracellular protein exchange especially at high filtration rates during lung injury. Our results suggest that 2 h after LPS application a transcellular active vesicular transport predominates. That might be due to that early point in time with a majority of endothelial cells alive and even boosting their activity. On the other hand, we also found strong evidence for a paracellular protein transport exclusively in the LPS group. So one might speculate that a paracellular transport could get overwhelming with higher LPS doses or with prolonged periods of LPS application.

The consequence of the enhanced transcellular and paracellular transport of albumin molecules was that the resulting air-blood barrier composed of the epithelium, the capillary endothelium, and the interstitial space was significantly thicker 2 h after LPS infusion compared with control conditions because of cellular and extracellular swelling.

As our standard for comparison, the development of lung edema in early acute lung injury was further calculated by width of alveolar septa as measured by intravital microscopy. These findings emphasize the electron microscopic results. In fact, intravital microscopy complements electron microscopy, since electron microscopy is not able to investigate more than one point in time, whereas intravital microscopy can create an almost continuous idea of the processes, however, with a lack in resolution. In choosing a time point for electron microscopy, we determined that 2 h after the onset of LPS application seemed to be a good compromise, considering that the upregulation of many inflammatory mediators can take hours, although the alveolar septum width data indicate that the first onset of pulmonary edema can occur as early as 30 min after LPS application or even earlier.

Accordingly, a slight but significant deterioration of the pulmonary diffusion features developed in the LPS group.

Neither albumin concentration in BAL nor lung tissue wet/dry weight ratio was sensitive enough to detect the onset of pulmonary edema formation. That might be due to a relatively low-dose LPS application that was chosen to keep the animals macrohemodynamically stable and therefore to minimize effects of filtration pressure on extravasation of albumin. Furthermore, the LPS dose was selected to be as low as possible to underline the sensitivity of the method.

It is known that tissue damage resulting in capillary leakage and edema generation is based on the accumulation and activation of inflammatory cells (19, 20) followed by the generation and release of the highly reactive peroxynitrite (6, 15, 54). Peroxynitrite activates the nuclear enzyme PARS via DNA damage (49, 50, 56). There is strong evidence that PARS activation is a key player in the genesis of acute lung injury, because it supports a vicious circle of further inflammatory cell accumulation through upregulation of adhesion molecules (19, 20) and it induces a massive and fatal energy depletion in affected endothelial cells (49, 50, 56). Therefore, PARS inhibition by 3-AB was chosen for an intervention supposed to protect the lung against LPS. 3-AB is a well-known competitive and nontoxic inhibitor of PARS (47, 48). The main findings are that PARS inhibition during systemic inflammation was able to normalize microanatomy and to prevent the increase of vesicle and macromolecule concentration in endothelial and epithelial cells. Furthermore, widening of the gas exchange barrier and enlargement of alveolar septum width as a result of LPS application were successfully treated with PARS inhibition. These improvements led to a normalization of the gas exchange.

In conclusion, the main goal of this study was to establish a new and sensitive technique that brings the high resolution of electron microscopy in line with the determination of microvascular macromolecular permeability in early acute lung injury. The availability of this technique will allow accurate assessment of physiological and pathophysiological microvas-
cular characteristics and will form the basis for evaluating novel treatment strategies in acute lung injury.

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