MICROHEMODYNAMICS AND LEUKOCYTE SEQUESTRATION AFTER PULMONARY ISCHEMIA AND REPERFUSION IN RABBITS

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Objective: **Investigation of leukocyte sequestration in alveolar capillaries and of microhemodynamic changes after pulmonary ischemia/reperfusion injury.** ***Methods:*** **The kinetics of leukocyte passage and the hemodynamics in pulmonary microcirculation were investigated in 16 rabbits by intravital microscopy. Mean red blood cell velocity and the number of sticking leukocytes were measured in pulmonary arterioles, venules, and capillaries after 1 hour of tourniquet ischemia and 10 minutes and 1 hour after reperfusion.** ***Results:*** **The decrease of red blood cell velocity after reperfusion was associated with a largely increased heterogeneity of blood flow. Immediately after the onset of blood flow, sequestered leukocytes were found in all microvascular segments. An increased number of leukocytes was present in arterioles, venules, and alveolar capillaries 10 minutes and 1 hour after reperfusion. Concomitantly, width of alveolar septa was increased while arterial oxygen tension was reduced, indicating the development of interstitial pulmonary edema.** ***Conclusion:*** **Leukocytes are sequestered after pulmonary ischemia and reperfusion not only in alveolar capillaries but also in arterioles and venules, and they may contribute to the development of reperfusion edema. (J Thorac Cardiovasc Surg 1998; 115:937-44)**

Ischemia-reperfusion injury of the lung is a mechanism of injury in various pulmonary diseases. Lung transplantation and cardiopulmonary bypass are clinical situations in which lung perfusion is temporarily interrupted and reperfusion injury occurs.[1] Acute lung injury was also observed after pulmonary embolectomy[2] or after reexpansion of atelectasis.[3] Resolution of vascular microthromboses seen in adult respiratory distress syndrome may also produce reperfusion injury.[4]

Increasing evidence exists that sequestration and activation of polymorphonuclear leukocytes during reperfusion represents the central event in the development of ischemia-reperfusion injury of the lung.[1, 5-9] Accumulation of leukocytes in the lungs, as evidenced by increased myeloperoxidase activity, starts within the first 30 minutes after reperfusion.[9] However, the mechanism by which neutrophils are initially sequestered and the anatomic site of sequestration are unclear. In histologic sections of the lung, leukocytes are found in alveolar septa and within alveoli 2 to 3 hours after ischemia and reperfusion.[6] Recent studies have suggested that the relationship between the size of neutrophils and the cross section of alveolar capillaries may be an important determinant of physiologic leukocyte retention in the lung.[10-14] The neutrophil is approximately 6.4 μm in diameter, but the average diameter of pulmonary capillaries is distinctly smaller (6.0 μm).[13] Hence neutrophil deformation is essential for maintaining flow through pulmonary capillaries.[13] Furthermore, the microvascular blood flow itself is a factor contributing to the process of leukocyte retention in pulmonary capillaries.[12, 15, 16] If activation of leukocytes with inflammatory agents causes the cells to become less deformable (stiffening) or to increase their volume (swelling), they might be additionally retained (i.e., sequestered) within the capillaries because of mechanical reasons.[10, 16, 17]

Various studies have demonstrated that adhesion

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molecules are involved in sequestration of leukocytes in the lung, particularly after ischemia and reperfusion.[5, 7] Leukocyte-endothelium interactions mediated by adhesion receptors in organs other than the lung have been observed only in postcapillary venules.[18, 19] In those organs, leukocyte emigration is thought to occur in three sequential steps during an acute inflammatory response.[19] The initial event is a slowing of leukocyte transit through the injured site by reversible loose adhesion between leukocytes and endothelium. This reversible adhesion appears as rolling of leukocytes along the vascular endothelium. This phenomenon was recently observed also in pulmonary arterioles and venules.[11, 12] The second step is further activation and firm adhesion of leukocytes. Finally, leukocytes extravasate along a chemotactic gradient. It has been proposed that this three-step model cannot be applied to the pulmonary circulation[16] because the primary site for margination and migration is in the capillaries, where the space constraints do not allow the leukocytes to roll and because neutrophil emigration out of the pulmonary circulation appears to be mediated by a CD11/CD18 adhesion molecule dependent and independent pathway.

However, in the lung, little experimental data are available that delineate the time course and the topographic distribution of leukocyte sequestration under the conditions of flow and their relationship to the development of tissue injury, especially after lung ischemia and reperfusion.

To resolve these questions, we investigated microhemodynamics and leukocyte kinetics in the pulmonary arterioles, venules, and alveolar capillaries and measured the thickness of alveolar septa after 1 hour ischemia and subsequent reperfusion using an intravital microscopic approach.[11, 12, 20, 21]

Methods

Eighteen adult male White New Zealand rabbits weighing 2500 to 3100 gm were used. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985). The animals were anesthetized with thiopental sodium (50 mg intravenously) followed by α-chloralose (50 mg/kg body weight intravenously). Piritramide (0.5 mg/kg intravenously) was given for analgesia and pancuronium bromide (0.3 mg/kg intravenously) for neuromuscular blockade. Tracheostomy was performed on the rabbits and they were ventilated with 33% oxygen at an inspiratory airway pressure of 8 mm Hg. To record systemic arterial and central venous pressures, catheters were advanced into the aorta and superior vena cava through the carotid artery and jugular vein. Samples for arterial blood gas analysis were taken (ABL, Radiometer, A/S, Copenhagen, Denmark). Respiratory rate was adjusted to establish mean arterial carbon dioxide tension of 35 to 40 mm Hg. After a small thoracotomy in the second intercostal space, a catheter was implanted into the pulmonary artery to record pulmonary artery pressure. After a second thoracotomy in the left fourth intercostal space close to the sternum, a tourniquet catheter was placed around the hilum of the left lung using a J-shaped steel cannula.

For implantation of a transparent window into the left thorax, 3 cm of the third and fourth rib of the left thorax were surgically removed. To prevent the exposed lung surface from drying or cooling, the membrane of the window was superfused with warmed and gassed Tyrode's solution. For intravital microscopy, the animal was placed in right lateral position under a modified Leitz-Orthoplan microscope (Leica, Wetzlar, Germany) on an electronic scanning table (Phytron, Gröbenzell, Germany). Techniques used for microhemodynamic measurements have been previously described in detail.[11, 12, 20] Microvascular blood flow in subpleural arterioles, venules, and alveolar capillaries was visualized by fluorescence microscopy after intravenous injection of fluorescein isothiocyanate (FITC)–labeled red blood (fluorescein isothiocyanate; Sigma, St. Louis, Mo.). Leukocytes were stained in vivo by bolus injection of Rhodamin 6G (Merck, Darmstadt, Germany). Green light–emitting FITC-labeled red blood cells (RBCs) and red light–emitting leukocytes were differentiated by using appropriate excitation and emission filters (L2 and N2 filter blocks; Leica, Wetzlar, Germany). Fluorescence images were monitored with a silicon-intensified target camera (C 2400; Hamamatsu, Herrsching, Germany) and recorded on videotape (AG 7350; Panasonic, Munich, Germany). To avoid respiratory movements of the lung surface video recordings were performed during inspiration periods of 5 seconds.

Quantification of microhemodynamics and leukocyte sequestration. Measurements were performed using an image analysis system (Optimas 3.0, Bioscan, Edmonds, Wash.). Inner diameters of subpleural arterioles and venules and the velocity of all labeled RBCs passing a predefined vessel cross section were measured as described previously.[11, 12, 20, 21] Sticking leukocytes (i.e., cells adherent to the inner vessel surface) were defined as cells not moving for at least 5 seconds. They are depicted as the number of leukocytes per wall surface area of the observed vessel. To determine the kinetics of RBCs and leukocytes in pulmonary capillaries, the subpleural wall of single alveoli was investigated. The boundaries of the alveolar wall were determined interactively using the image analysis system. To determine the mean RBC velocity in the capillary network, the velocity of each labeled RBC crossing the boundary and entering the network was measured. Analogous to arterioles and venules, the number of leukocytes within the capillary network and not moving for at least 5 seconds was

Table I. *Macrohemodynamics and blood gases*

	Baseline	*Ischemia*	*10 min after reperfusion*	*1 hr after reperfusion*
MAP (mm Hg)	83.1 ± 3.1	83.5 ± 3.3	80.6 ± 3.5	79.2 ± 2.6*
	(81.9 ± 1.9)		(88.5 ± 4.0)	(90.5 ± 4.1)
PAP† (mm Hg)	12 ± 0.5	14.4 ± 1.4	12.5 ± 1.4	13.8 ± 1.3
	(12.2 ± 0.9)		(14.4 ± 1.1)	(13.6 ± 1.2)
CVP (mm Hg)	2.6 ± 0.5	2.6 ± 0.4	2.6 ± 0.4	2.8 ± 0.4
	(2.9 ± 0.6)		(2.2 ± 0.6)	(3.5 ± 1.2)
Pa_{O_2}	152 ± 15.9	104 ± 13.6	111 ± 9.3*	118 ± 8.0*
	(154 ± 6.5)		(144 ± 10.3)	(142 ± 8.5)
Pa_{CO_2}	35.2 ± 3.5	47 ± 6.6	60.1 ± 7.9‡	59.9 ± 7.8‡
	(38.1 ± 2.5)		(41.6 ± 2.7)‡	(47.6 ± 2.6)‡

Values are mean ± SEM; ischemia ($n = 9$) and controls ($n = 9$) are in parentheses.

*$p = 0.04$ vs control.

†$n = 7$ because of technical problems.

‡$p < 0.05$ vs baseline.

determined and expressed as number of sticking leukocytes per alveolar wall area. In addition, the width of subpleural alveolar septa was measured. For this, the boundaries of the alveoli were estimated manually based on the greatest changes of grey level section perpendicular to the course of alveolar septa. Then the closest distances between the investigated alveolus and all adjacent alveoli were measured at least three times.

Experimental protocol. After a preparation period of approximately 2 hours, the animals were placed under the microscope and allowed to stabilize for 15 minutes and ventilated at an inspiratory oxygen concentration of 100%. Baseline macrohemodynamic and blood gas analyses were performed. Heparin (150 IU/kg) was given intravenously to inhibit intravascular coagulation during ischemia. Then, if meeting the inclusion criteria during baseline (i.e., stable macrohemodynamic conditions [mean arterial pressure > 80 mm Hg], unaltered gas exchange [arterial oxygen tension > 450 mm Hg], macroscopically homogenous perfusion of the lung, and no signs of injury), the animals were randomly assigned to the ischemia group or the control group. In the ischemia group, the tourniquet placed around the lung hilus was tightened and perfusion and ventilation were stopped immediately. After 30 minutes, FITC-labeled RBCs were injected intravenously and allowed to recirculate. One hour after induction of ischemia, macrohemodynamic measurements were performed, the tourniquet was opened, and the left lung was ventilated and reperfused again. To stain the leukocytes, rhodanine 6G was injected, and 10 minutes after reperfusion in vivo microscopy was performed. Animals in the control group were treated identically except for tightening of the tourniquet. Video recordings were taken during approximately 15 minutes at 10 minutes and 1 hour after reperfusion. To investigate the microhemodynamics and leukocyte kinetics, video recordings were taken during inspiration periods prolonged to 10 seconds. To investigate the heterogeneity of reperfusion and leukocyte sequestration, videorecordings of four different areas on the lung surface, two on the upper and two on the lower lung lobe, were taken. Within each area, four different alveolar wall areas were investigated. Pulmonary arterioles and venules were predominantly located on the edge of the left upper lung lobe. In each animal one to three arterioles and venules were investigated. To ensure that identical alveoli and blood vessels were recorded in the different phases, respective x-y coordinates of the electronic scanning table were registered and recalled later on.

Statistics. All data are given as mean ± standard error of the mean. Data were tested for normality using the Kolmogorov Smirnov test. If the normality test was passed, the *t* test was used for statistical comparison of ischemia and control group and for comparison of microhemodynamic data 10 minutes and 1 hour after reperfusion, the paired *t* test was used. Sequential macrohemodynamic and blood gas data were tested using one-way repeated-measures analysis of variance followed by Bonferroni *t* test for multiple comparisons if significant differences were detected. If the data varied significantly from normal distribution, the respective nonparametric tests were used. Statistical analysis was performed using the computer program SigmaStat (Sigma, St. Louis, Mo.). To investigate the heterogeneity of microhemodynamics or leukocyte sequestration, the coefficient of variation (CV) between the different alveolar wall areas within the animals was calculated.

Results

Macrohemodynamics and blood gases. Table I summarizes the results of macrohemodynamic and blood gas measurements. In the ischemia and the control groups central venous pressure and pulmonary artery pressure were not significantly different during the different phases of the experiments. Mean arterial pressure was significantly lower at 1 hour after reperfusion in the ischemia group. Arterial oxygen tension was significantly lower in ischemia group at 10 minutes and 1 hour after reperfu-

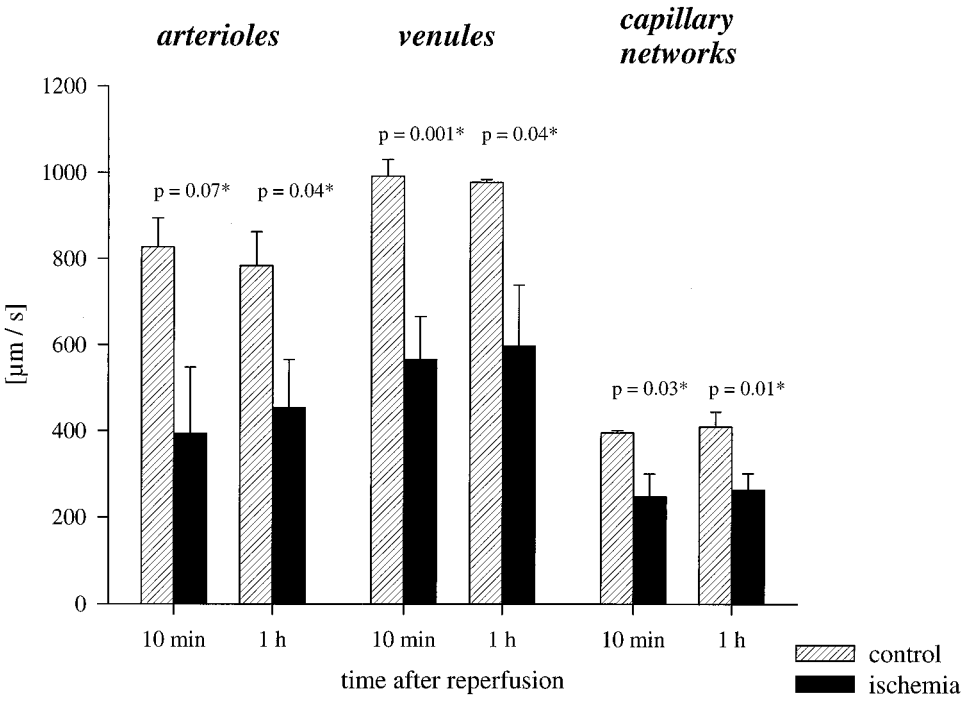

Fig. 1. Mean RBC velocity in arterioles (ischemia: $n = 7$; control: $n = 8$), venules (ischemia: $n = 8$; control: $n = 8$), and capillary networks (ischemia: $n = 7$; control: $n = 8$) at 10 minutes and 1 hour after reperfusion. Values are means ± SEM. *Ischemia versus control.

sion. In the ischemia group and the control group arterial carbon dioxide tension was significantly increased at 10 minutes and 1 hour after reperfusion compared with baseline.

Microhemodynamics and leukocyte kinetics. During ischemia, video recordings were regularly taken. No movement of FITC-labeled RBCs or ventilation-dependent movement of the lung surface was detected, indicating that the left lung was not perfused or ventilated during that time. A few seconds after opening the tourniquet, the first moving RBCs and leukocytes were normally seen.

After 10 minutes of reperfusion, the first systematic video recordings were performed. Considerable differences of mean RBC velocity were measured in arterioles of different animals, ranging from 7 µm/sec to 793 µm/sec (CV 96%) and to a lesser extent in alveolar capillary networks, ranging from 56 µm/sec to 436 µm/sec (CV 59%). In pulmonary venules interindividual variance of RBC velocity 10 minutes after reperfusion was lower (range 311 µm/sec to 1045 µm/sec, CV 50%).

Compared with the control group, RBC velocity was significantly lower in capillary networks and pulmonary venules (Fig. 1).

Heterogeneity of capillary reperfusion was significantly higher in the ischemia group, as indicated by the CV of mean RBC velocity within the animals (ischemia 108% ± 47% vs control 21% ± 4%; $p = 0.04$).

The first nonmoving leukocytes were observed immediately after onset of blood flow. Ten minutes after reperfusion, the average number of sticking leukocytes was significantly higher in pulmonary arterioles, venules, and alveolar capillaries compared with controls (Fig. 2). Sixteen alveoli of two animals were not perfused 10 minutes after opening the tourniquet. No labeled leukocytes were seen in 15 of these alveoli. CV of the number of sticking leukocytes within animals was not statistically different between groups (ischemia 72% ± 23% vs control 38% ± 5%).

One hour after reperfusion, the mean RBC velocity slightly increased in arterioles, venules, and alveolar capillaries (Fig. 1). In arterioles and capillaries, velocity increased predominantly in primarily low-perfused vessels or alveoli and decreased in some of the arterioles or alveoli with primarily high RBC velocity. Therefore the interindividual difference decreased (arterioles range 30 µm/sec to 733

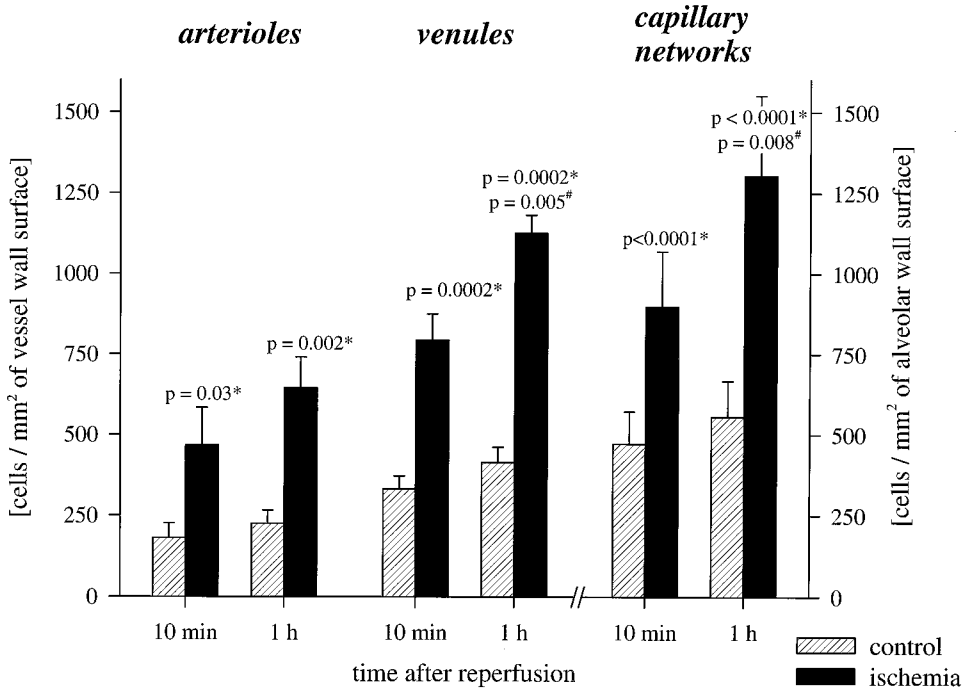

Fig. 2. "Sticking" leukocytes in arterioles (ischemia: $n = 7$; control: $n = 8$), venules (ischemia: $n = 8$; control: $n = 8$), and capillary networks (ischemia: $n = 7$; control: $n = 8$) at 10 minutes and 1 hour after reperfusion. Values are means ± SEM. *Versus control; #1 hour vs 10 minutes.

μm/sec, CV 61%; capillaries range 171 μm/sec to 416 μm/sec, CV 38%). In venules, interindividual variability did not change notably (range 55 μm/sec to 1010 μm/sec, CV 67%). However, in all segments of the pulmonary microcirculation measured, mean RBC velocity was still significantly lower than in the control group 1 hour after reperfusion (Fig. 1). Again, heterogeneity of RBC velocity in alveolar capillary networks was significantly higher after ischemia compared with controls (ischemia 58% ± 17% vs control 21% ± 3%; $p = 0.04$).

In the ischemia group the number of sticking leukocytes significantly increased in capillary networks and in pulmonary venules 1 hour compared with 10 minutes after reperfusion (Fig. 2). Sticking leukocytes in arterioles, venules, and capillary networks were still significantly increased at 1 hour after reperfusion if compared with controls (Fig. 2). Again, CV of the number of sticking leukocytes within animals was not statistically different between groups (ischemia 35% ± 8% vs control 34% ± 4%).

Coinciding with reperfusion and leukocyte sequestration, the width of alveolar septa was observed to be significantly broader 10 minutes and 1 hour after reperfusion in comparison with the width of alveolar septa measured in the respective control (10 minutes ischemia 22 ± 1.2 μm vs control 15 ± 0.6 μm, $p = 0.0002$; 1 hour ischemia 20 ± 1.5 μm vs control 15 ± 0.6 μm, $p = 0.01$).

Discussion

This article describes the microhemodynamic changes and leukocyte kinetics after 1 hour of pulmonary ischemia and 1 hour of reperfusion. In the early phase, 10 minutes after reperfusion, the results show considerable intraindividual and interindividual heterogeneities of blood flow velocity in all types of pulmonary microvessels. Concomitant to the onset of blood flow, the first sticking leukocytes were observed in pulmonary arterioles, venules, and alveolar capillaries. One hour after reperfusion, a further significant increase of sticking leukocytes was found in venules and capillaries. Mean RBC velocity was lower after ischemia and reperfusion at any time and in all microvascular segments.

Model. Intravital microscopy has often been used to investigate ischemia-reperfusion injury in the organs of the systemic circulation. It allows simulta-

neous analysis of blood flow and leukocyte behavior in all types of microvessels, including arterioles, venules, and capillaries. Leukocytes are labeled in vivo after injection of the fluorescent dye rhodamine 6G. With this dye, all intravascular leukocytes, including lymphocytes, neutrophils, other granulocytes, and platelets, are fluorescent for at least 90 minutes, avoiding uncontrolled activation of the cells caused by in vitro separation.

Macrohemodynamics and gas exchange. Macrohemodynamic conditions and gas exchange at baseline and in the control group correspond to previously reported data of the same model. The mean aortic pressure was significantly lower 1 hour after reperfusion in the ischemia group compared with the control group, indicating a slight trend for increasing blood pressure in the control group and a trend to hypotension in the ischemia group over time. During ischemia and after reperfusion, arterial oxygen tension was significantly lower than control. Two different mechanisms may be responsible for this finding. First, the heterogeneity of blood flow after reperfusion of the lung may result in a mismatch of ventilation and perfusion in that lobe. Second, ischemia and subsequent reperfusion is known to induce an acute inflammatory response. An interstitial edema may be the consequence. The significantly broader width of subpleural alveoli observed 10 minutes and 1 hour after reperfusion is a direct indicator of an interstitial lung edema. As a result, diffusion of oxygen may be limited and arterial oxygen tension may fall.

Microhemodynamics. Hemodynamic parameters play a pivotal role in the physiology and pathophysiology of the lung, inasmuch as pronounced flow heterogeneities alone or together with ventilation heterogeneities may result in ventilation/perfusion mismatch and therefore in an impaired gas exchange. After ischemia and reperfusion, several hemodynamic changes are described including "no reflow" and reflow heterogeneities.[22] Histologic, electron microscopic, and intravital microscopic analyses in other organs have shown that the reason for the extreme heterogenous perfusion after ischemia includes swelling of microvascular endothelium, thrombosis of microvessels, plugging of capillaries by leukocytes, impairment of microvascular blood fluidity, and increased hydraulic resistance.[22] All these phenomena may also take place in the lung after ischemia and reperfusion and may primarily increase blood flow heterogeneity. An early increase of vascular resistance is not expected because all factors leading to a higher resistance to flow should result in an early cessation of regional blood flow and a redistribution of blood flow to initially unperfused capillaries or to regions exhibiting lower vascular resistance. Our results show considerable individual and interindividual heterogeneity of blood flow velocity 10 minutes after the start of reperfusion, especially in pulmonary arteries and alveolar capillaries. The lower interindividual variance of blood flow in venules is explained by the lower downstream flow resistance in these vessels. A relative homogenization of blood flow velocity was observed 1 hour after reperfusion, indicating that flow restrictions had been resolved. However, RBC velocity in arterioles, venules, and capillaries was still significantly lower than control.

Leukocyte kinetics. Immediately after the start of reperfusion, the first stagnant leukocytes were observed in all pulmonary vessels investigated. Ten minutes after reperfusion, the number of sticking leukocytes was significantly increased in pulmonary arterioles, venules, and alveolar capillaries. An additional increase of sticking leukocytes was observed after 1 hour in all parts of the pulmonary microcirculation, reaching statistical significance in pulmonary venules and alveolar capillaries. The previously reported difference in the number of leukocytes sticking in arterioles compared with venules could be confirmed in this study.[11, 12, 21] This difference remains in the ischemia groups. Because RBC velocity was higher in venules than in arterioles, the most plausible explanation is a difference of the adhesive properties of the vascular endothelium. This compares well to the findings in microvessels of the systemic circulation. There, leukocyte sticking is almost exclusively observed in postcapillary venules. The prevalence for adherence to venular endothelium is explained by the expression of adhesion receptors predominantly in venules. In the lung microcirculation, leukocyte sticking occurs also in arterioles, a feature already suggested by Staub, Schultz, and Albertine,[23] however, first demonstrated by Kuebler[11] and Kuhnle[12] and recently corroborated by Gebb and colleagues.[24] Besides differences between endothelium in arterioles of the pulmonary and systemic circulation, the much lower shear rate in pulmonary arterioles may facilitate leukocyte sticking.[12] Quantitatively, the largest population of sequestered leukocytes are the cells sequestered in the capillary networks of lung alveoli, inasmuch as the number and the vascular surface of pulmonary capillaries far exceeds the number and

surface of pulmonary arterioles and venules. Here the mechanisms and potential consequences of leukocyte sequestration in the lung after ischemia and reperfusion should be discussed. This study shows that in addition to leukocyte sequestration in capillaries, sticking of leukocytes in arterioles and venules occurs. If it is assumed that sticking is an adhesion-mediated phenomenon, it may be concluded that endothelial adhesion molecules in the lung are constitutively expressed or are up-regulated during ischemia and initial reperfusion. Because sticking leukocytes were also observed in the control group, both may be true. In addition, low blood flow velocity and thus low shear forces may promote leukocyte adhesion.[12, 15] The same might be true for pulmonary capillaries, but to our knowledge adhesion-mediated leukocyte endothelium interactions in capillaries have been proven neither in alveolar capillaries nor in the capillaries of the systemic circulation. However, extensive work has been carried out to investigate the role of mechanical properties explaining leukocyte retention and sequestration in alveolar capillaries.[16] Unquestionably, the discrepancy between capillary internal diameter and leukocyte diameter, and therefore the need of the leukocyte to deform to pass through the capillary, is a sufficient explanation for physiologic leukocyte retention in capillaries. Even more, this effect should be pronounced if leukocytes become activated and therefore less deformable and if endothelial cells become injured and swollen. It seems possible that under such conditions leukocytes may be permanently trapped in alveolar capillaries and may contribute to reperfusion injury. On the other hand, various studies have shown adhesion molecules being involved in sequestration of leukocytes in the lung, even after ischemia and reperfusion. Some of those studies could demonstrate a reduction of reperfusion injury if adhesion molecules were blocked.[5, 7, 25] Because it seems unlikely that these protective effects of antiadhesion therapy target only leukocyte-endothelial interactions in venules or arterioles, it may be speculated that adhesion molecule–dependent sequestration[21] or emigration of leukocytes occurs in alveolar capillaries as well.

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