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RESEARCH ARTICLE

Pulmonary endothelial permeability and tissue fluid balance depend on the viscosity of the perfusion solution

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Rowan SC, Rochfort KD, Piouceau L, Cummins PM, O'Rourke M, McLoughlin P. Pulmonary endothelial permeability and tissue fluid balance depend on the viscosity of the perfusion solution. *Am J Physiol Lung Cell Mol Physiol* 315: L476–L484, 2018. First published May 24, 2018; doi:10.1152/ajplung.00437.2017.— Fluid filtration in the pulmonary microcirculation depends on the hydrostatic and oncotic pressure gradients across the endothelium and the selective permeability of the endothelial barrier. Maintaining normal fluid balance depends both on specific properties of the endothelium and of the perfusing blood. Although some of the essential properties of blood needed to prevent excessive fluid leak have been identified and characterized, our understanding of these remains incomplete. The role of perfusate viscosity in maintaining normal fluid exchange has not previously been examined. We prepared a high-viscosity perfusion solution (HVS) with a relative viscosity of 2.5, i.e., within the range displayed by blood flowing in vessels of different diameters in vivo (1.5–4.0). Perfusion of isolated murine lungs with HVS significantly reduced the rate of edema formation compared with perfusion with a standard solution (SS), which had a lower viscosity similar to plasma (relative viscosity 1.5). HVS did not alter capillary filtration pressure. Increased endothelial shear stress produced by increasing flow rates of SS, to mimic the increased shear stress produced by HVS, did not reduce edema formation. HVS significantly reduced extravasation of Evans blue-labeled albumin compared with SS, indicating that it attenuated endothelial leak. These findings demonstrate for the first time that the viscosity of the solution perfusing the pulmonary microcirculation is an important physiological property contributing to the maintenance of normal fluid exchange. This has significant implications for our understanding of fluid homeostasis in the healthy lung, edema formation in disease, and reconditioning of donor organs for transplantation.

edema; endothelium; isolated perfused lung; permeability

INTRODUCTION

Normal tissue function requires that water and soluble constituents of blood, including oxygen and carbon dioxide, can be exchanged with the interstitial fluids in the perivascular region. This exchange must be achieved while preventing excessive fluid leak into the tissues. Failure to maintain normal fluid exchange leads to edema formation and impairment of tissue function, for example failure of gas exchange in pulmonary edema.

Fluid exchange depends on the balance of hydrostatic and oncotic pressure differences between the lumen of the microvasculature and the interstitial space, and the selective permeability of the endothelium to molecules of different sizes, as described in the classic Starling hypothesis (27). The essential roles of the normal electrolyte composition, pH, nutrient, hormonal and colloid osmotic pressure of the perfusion solution in maintaining vascular function have been well characterized in the classic isolated organ preparations such as the isolated heart, lung, and kidney, and more recently in ex vivo perfusion protocols used to recondition donor lungs before transplantation (6, 16, 22, 25, 27, 35). However, these isolated organ preparations develop edema after relatively brief periods (hours). To further increase the period for which isolated organs can be maintained viable, nonphysiological modifications of classic perfusion media have been introduced such as increasing concentrations of albumin and globulins substantially above normal in vivo values to increase colloid osmotic pressure, and addition of pharmacological vasodilators and oxidant scavengers (25, 35). Nonetheless, development of edema after relatively restricted periods of time remains a problem (6, 35).

Whole blood is significantly better than acellular artificial perfusates at preventing edema formation and preserving normal function in isolated lungs and other organs, and better than artificial perfusates with added erythrocytes (24, 25, 37). These findings suggest that there are physiological properties of blood that are important in maintaining normal fluid exchange in the microvasculature of the intact organ that have not yet been identified.

One of the properties of whole blood that differs markedly from artificial perfusates is viscosity. The potential contribution of perfusate viscosity to the maintenance of normal fluid balance in the intact pulmonary microcirculation has not been previously specifically investigated. The viscosity of blood is complex and depends upon shear rate, local hematocrit, and the diameter of the vessel through which it is passing (14, 18). This non-Newtonian behavior is a consequence of the cells suspended within the plasma and results in a variation in the relative viscosity of blood in vivo from a minimum value equal to that of cell free plasma (~1.5) in some regions of the microvasculature up to values of ~4.0 in large arteries and veins (14, 18). Thus, when cell free artificial perfusates similar

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to plasma are used in isolated organs, or perfusates with substantially lower hematocrits than normal blood, the viscosity is lower than that normally present in most vessels of the pulmonary circulation in vivo, so that shear stresses and pressure gradients throughout the circulation deviate significantly from the normal in vivo values.

To our knowledge, the influence that the viscosity of a perfusing solution has on the physiological regulation of endothelial barrier function and fluid filtration in the intact pulmonary circulation has not been previously reported. We found that perfusion of the isolated ventilated lung with a solution that had a higher relative viscosity (2.5) than that of plasma (1.5), but within the range observed in blood in vivo (1.5–4.0), reduced fluid filtration and edema formation and substantially extended the duration of normal lung function by enhancing endothelial barrier function. This novel finding has important implications for our understanding of the maintenance of fluid balance in the normal microcirculation and edema formation in pathophysiological conditions.

METHODS

The isolated ventilated perfused mouse lung was used to assess vascular function, as previously described (11). Lungs were isolated postmortem from specific pathogen-free adult male C57Bl6 mice (10–12 wk old). Euthanasia was performed by exsanguination under deep anesthesia (pentobarbitone sodium, 60 mg/kg ip). All procedures on mice were approved by the University College Dublin Animal Research Ethics Committee.

The standard solution (SS) used for perfusion consisted of Dulbecco's Modified Eagle's Medium (DMEM, Sigma, Dublin, Ireland, catalog no. D6046) with Ficoll molecular mass 70 kDa (Sigma, Dublin, catalog no. F2878) added (4.0 g/100 ml) as a substitute for albumin, and had a relative viscosity of 1.5 (Table 1). A higher-viscosity solution (HVS) was used in some lungs consisting of SS with high-molecular-mass (400 kDa) Ficoll (Sigma, catalog no. F4375) added (3.25 g/100 ml) to produce a relative viscosity of 2.5 (Table 1). Finally to control for the associated increase in solution osmolality observed in the HVS we produced a third solution (6% wt/vol Ficoll PM70, Sigma) that matched the osmolality of the HVS through the addition of a higher percentage of low-molecular-mass Ficoll PM70 but had a substantially lower viscosity (Table 1). We used Ficolls in these solutions due to the previously reported innate immunity of rodents to dextrans (17, 20, 29). In pilot experiments we found that the use of dextrans caused rapid onset (within 10–15 min) of gross pulmonary edema in the isolated mouse lung (data not shown), a finding compatible with an immediate immune response to dextrans.

The viscosity of each perfusion solution was measured at 37°C using an Ostwald capillary viscometer.

The viscosity of each fluid was also measured over a range of physiological shear rates (300–3,000 s^{-1}) using a plate-plate rheometer (DHR2, TA Instruments), which demonstrated that the viscosity of the solutions was independent of shear rate (data not shown). Perfusate osmolality was measured using a vapor pressure osmometer (VAPRO, model 5520, Wescor). Table 1 indicates the characteristics of the three perfusion solutions.

Following an initial period of perfusion to allow stabilization, lungs were perfused at increasing flows (1.0, 2.0, 3.0, 4.0, and 5.0 ml/min, each for 5 min) to generate a pressure-flow curve and then returned to a steady-state low flow rate (LFR) of 2 ml/min, which was continued until edema developed (peak airway pressure >5.5 mmHg) or 180 min had elapsed.

Increasing the viscosity of the perfusion solution (HVS) inevitably increased the endothelial shear stress, since endothelial shear in a cylindrical pipe is linearly related to viscosity if flow and lumen radius are constant. Thus to determine whether any effect observed in the HVS solution might have been caused by increased endothelial shear, we also examined the time to edema development in a separate group of lungs perfused with SS but at higher flow rate (3.4 ml/min). The fold increase in this flow rate over LFR (3.4 ml/min:2.0 ml/min) equaled the fold increase in the viscosity of HVS compared with SS (2.5:1.5) and would therefore produce a similar increase in endothelial shear stress if vessel diameter were equal in these two flow conditions. In pilot analyses, we examined the effect of SS HFR on the shear stress-dependent phosphorylation of VEGFR2 (33) and found that it was similar to that produced by HVS LFR (data not shown).

Values for alpha (α), representing the distensibility of the pulmonary vessels, and R_0, representing the hemodynamic resistance of the undistended pulmonary vascular bed, were obtained by using the model of Linehan and colleagues (23) to describe the pressure-flow curve data generated in the isolated perfused lung experiments. In brief, this characterizes the pressure-flow relationship in the pulmonary circulation, which is formed from easily distensible vessels, as follows:

$$P_{pa} = \frac{\left[(1 + \alpha P_v)^5 + 5\alpha R_0 Q\right]^{1/5} - 1}{\alpha} \quad (1)$$

where P_{pa} is the pulmonary artery pressure, P_v is the venous outflow pressure (left atrial pressure), and Q is the total flow through the lungs. α is an index of the distensibility of the pulmonary vessels, which is assumed to be the same for all the vessels, and is described by the following equation:

$$D_p = (1 + \alpha P) \cdot D_0 \quad (2)$$

where D_p is the diameter of the vessel at any given distending pressure P, and D_0 is the diameter of the vessel when the distending pressure is zero. R_0 is the vascular resistance of the pulmonary circulation when there is no distending pressure within the vascular bed, i.e., when Q approaches 0. R_0 is directly proportional to the viscosity of the perfusing fluid and to the geometric structure of the pulmonary circulation (e.g., the radii of all the individual vessels, the lengths of the individual segments, branching patterns, etc.). Thus

$$R_0 = \mu R_0(H_2O) \quad (3)$$

where μ is the viscosity of the perfusing solution expressed relative to water (relative viscosity) and $R_0(H_2O)$ is the R_0 that would be measured using *Eq. 1* if the circulation were perfused with a solution whose viscosity equaled that of water (23). Since we had measured the viscosity of the perfusion solutions and expressed them relative to

Table 1. *Ficoll content, viscosity, and osmolality of the three solutions used to perfuse isolated lungs*

	Standard Solution (SS)	High-Viscosity Solution (HVS)	6% Ficoll Solution
Ficoll (MM 70 kDa)	4% (wt/vol)	4% (wt/vol)	6% (wt/vol)
Ficoll (MM 400 kDa)		3.25% (wt/vol)	
Relative viscosity	1.5	2.5	1.8
Osmolality, mosmol/kgH_2O	326	331	331

Solution viscosity expressed relative to the viscosity of H_2O. MM, molecular mass.

water, we were able to calculate $R_0(H_2O)$ from *Eq. 3*, giving us an index of changes in the geometric properties of the pulmonary circulation that contributed to the changes in vascular resistance caused by the different perfusion solutions. $R_0(H_2O)$ thus reflects vascular hindrance, i.e., the vascular resistance caused by the characteristics of the blood vessels excluding the effects of solution viscosity on the pressure-flow curves (13). It is worth noting that these geometric properties can vary acutely, e.g., due to vasoconstriction or vascular recruitment.

In some experiments capillary pressure (P_{cap}) was determined during the period of steady-state flow that followed the collection of the data to characterize the pressure-flow curve in each lung. The double-occlusion technique was used to determine P_{cap}, i.e., by simultaneous occlusion of arterial and venous cannulas at end expiration as previously described (10, 32). Under constant-flow conditions, capillary pressure depends on the arterial inflow pressure (P_{pa}), the venous outflow pressure (Pv), the arterial resistance upstream of the capillaries (R_a), and the venous resistance downstream of the capillaries (R_v). Then:

$$R_a = (P_{pa} - P_{cap})/Q \quad (4)$$

and

$$R_v = (P_{cap} - P_v)/Q \quad (5)$$

Since total resistance (R_t) is the sum of R_a and R_v, we can rearrange to give

$$P_c = P_{pa} + (R_a/R_t)(P_{pa} - P_v) \quad (6)$$

Thus the capillary pressure under steady-state conditions depends on P_{pa}, the proportion of the total resistance that lies in the arterial segment (R_a/R_t), and the pressure gradient across the vascular bed ($P_{pa} - P_v$).

For direct assessment of edema formation and vascular permeability, a separate series of experiments was undertaken in which the lungs were perfused with either SS or HVS with Evans Blue-labeled albumin added (0.5% wt/vol, Sigma). At the end of the period of perfusion, the vasculature was perfused with saline for 5 min (2 ml/min) until the draining perfusate was clear, so that only extravasated Evans Blue-labeled albumin remained in the lungs. Wet weight: dry weight ratio was determined, and formamide (≥99.5%, Sigma) was then added to each dried lung and incubated at 70°C for 1 h to extract Evans Blue dye. The lung was then homogenized, the homogenate cleared by centrifugation, and the concentration of Evans Blue determined by absorbance at 620 nm and expressed per milligram of lung dry weight (39).

Bovine pulmonary artery endothelial cells (BPAECs, Cell Applications) were used to examine permeability in monolayers in vitro. Cells were cultured in bovine endothelial cell complete growth-based medium (Cell Applications) for all experimental procedures and used at passage 6. Shear stress was applied using an orbital rotator, as previously described (15). Cells were seeded on six-well plates (5,000 cells/cm^2) and grown to confluence in HVS before the plates were rotated for 24 h at a calculated shear stress of 10 dyn/cm^2 to establish a barrier mimicking in vivo conditions. Control cells were maintained in static conditions (i.e., no shear stress) with HVS for 24 h and then had their media replaced with fresh HVS for the experimental duration.

Following each of these protocols, cells were harvested for analysis by immunoprecipitation (IP) in conjunction with Western blot analysis or for transendothelial permeability assay using FITC-labeled dextran as previously described (30). Details of the primary and secondary antibodies used are provided in Table 2.

Statistical analysis. Statistical analysis was performed using SPSS Statistics (IBM). All normally distributed data are presented as means ± SD. Statistical significance of the difference between means was determined using unpaired *t*-tests. When multiple comparisons of means were undertaken, the Holms-Sidak step-down correction was used (26).

Where data appeared nonnormally distributed, they are presented as medians [±interquartile ranges (IQR)], and the statistical significance of the difference between medians was tested using the Mann-Whitney *U*-test. When multiple comparisons of means were undertaken, the Holm-Sidak step-down correction was used (26).

In all experiments $P < 0.05$ was considered statistically significant; when $P > 0.001$, the exact *P* value is shown.

RESULTS

Perfusion of an isolated lung with a high viscosity solution protects against edema. Following the initial perfusion at progressively increasing flow rates (1–5 ml/min) to obtain data for the pressure-flow curve, isolated lungs subsequently perfused with SS at low flow rate (LFR, 2 ml/min) showed a period of stable peak airway pressures (Fig. 1*A*). However, after this stable interval, peak airway pressure increased progressively with increasing duration of perfusion (Fig. 1, *A* and *C*), a consequence of reduced compliance caused by interstitial fluid accumulation (8). In contrast, peak airway pressure remained stable for 3 h in lungs perfused with HVS at the same low flow rate (Fig. 1, *B* and *C*).

To see if the protective effect of HVS perfusion could be explained by increased endothelial shear stress, we examined the effect of increasing shear stress to a similar extent (without increasing viscosity) by increasing the perfusion rate of SS (SS-HFR) to 3.4 ml/min (See METHODS for details). This higher flow rate did not increase the time to edema development (Fig. 1*C*). This finding is compatible with previous reports in isolated vessels showing that increased flow is either without effect on vascular permeability, or increases it (1, 21).

In a further separate series of experiments ($n = 6$), we examined perfusion at low flow rate (2 ml/min) with a solution containing Ficoll PM70 at a concentration of 6% (wt/vol), which had an osmolality identical to that of HVS (see Table 1). This produced a small increase in relative viscosity compared with SS (from 1.5 to 1.8) but did not lead to any increase in the median time (IQR) to edema formation in these lungs, 139 (136–154) min, when compared with lungs perfused with SS (Fig. 1*C*). These results demonstrate that the protective action

Table 2. *Western blot analysis primary antibodies and associated secondary antibodies*

	Primary Antibody				Secondary Antibody				
	Dilution	Source	Code	Lot	Dilution	Anti-	Source	Code	Lot
PO_4-Tyr	1:1,000	Invitrogen	13–6600		1:2,000	Mouse	Cell Signaling	7076	31
Occludin	1:1,000	Invitrogen	33–1500		1:2,000	Mouse	Cell Signaling	7076	31
PO_4-eNOS (s1177)	1:1,000	Cell Signaling	9570	4	1:2,000	Rabbit	Cell Signaling	7074	26
eNOS	1:200	BD Biosciences	610297	6036940	1:2,000	Rabbit	Cell Signaling	7074	26

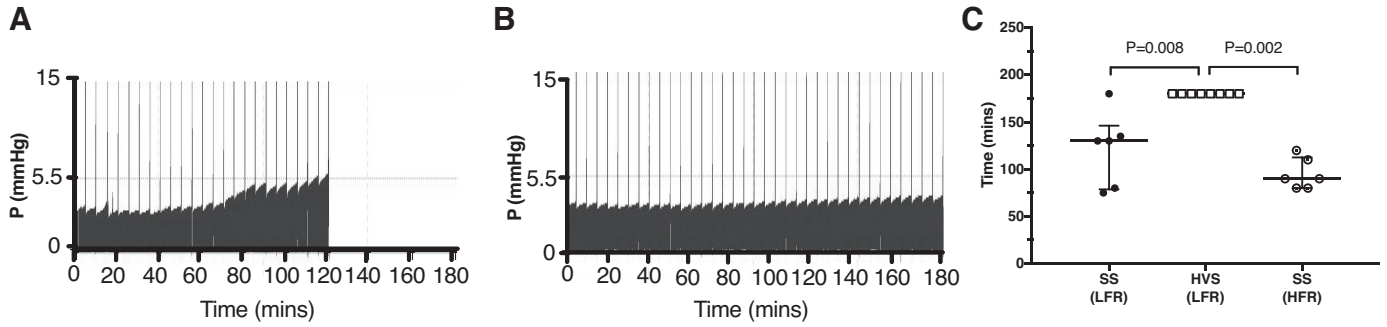

Fig. 1. Sample airway pressure (P) records from lungs perfused with either standard solution (SS) at low flow rate (*A*) or high-viscosity solution (HVS) at the same low flow rate (*B*). *C*: median (interquartile range) experimental duration in lungs perfused with SS at a low flow rate (SS-LFR), HVS at low flow rate (HVS-LFR), or SS at high flow rate (SS-HFR). *P* values indicate statistical significance of the difference between groups (Mann-Whitney *U*-test with Holm-Sidak correction).

of HVS against edema formation was not due to the small increase in osmolality caused by the addition of the high-molecular-mass Ficoll PM400.

Lungs perfused with SS cleared slowly of blood in an uneven, patchy manner with small areas of the lung persistently retaining visible blood, especially in more peripheral regions, suggesting heterogeneity of perfusion. In contrast, lungs perfused with HVS cleared more quickly of blood and became uniformly white, indicating more even distribution of perfusion.

Effect of HVS on pulmonary arterial pressure, vascular resistance, and capillary pressure. Following the initial generation of a pressure-flow curve in each lung, P_{pa} early during the steady flow condition, before any increase in peak inspiratory pressure or evidence of edema formation (60 min after initiation of perfusion), showed that HVS (LFR) perfusion did not cause a substantial change in steady-state P_{pa} when compared with SS (LFR) (Fig. 2*A*).

It is worth noting that although there was not a statistically significant difference ($P = 0.095$) between the SS (LFR) and HVS (LFR) groups, there is a single very high value in the SS (LFR) group. Grubb's test suggests that it was an outlier ($P = 0.003$) and when that outlier was omitted from the data the HVS (LFR) P_{pa} was significantly higher ($P = 0.004$) than that in the SS (LFR) group. Nonetheless, the difference in mean P_{pa} (~0.6 mmHg) between the two groups was much smaller than would be expected given the large increase in viscosity of the HVS solution (Fig. 2*A*). This observation suggests that perfusion with HVS (LFR) reduced pulmonary vascular resistance compared with perfusion with SS (LFR). In keeping with this, median $R_0(H_2O)$ (IQR) in lungs perfused with HVS was 1.67 (1.49–2.12) $mmHg \cdot ml^{-1} \cdot min^{-1}$, significantly lower ($P = 0.001$) than the median $R_0(H_2O)$ in those perfused with SS, which was 2.73 (2.35–3.56) $mmHg \cdot ml^{-1} \cdot min^{-1}$. Thus the intrinsic resistance of the pulmonary circulation independent of the viscosity of the perfusion solution (vascular hindrance) was reduced by HVS. This is compatible with the previously reported action of increased viscosity in the systemic circulation (13).

Additionally, mean P_{pa} during the steady-state HVS perfusion was always lower than that measured at the same flow during the initial pressure-flow curve in that lung (Fig. 2, *B* and *C*). This demonstrates a further reduction in vascular resistance compatible with flow-induced vasodilatation, as previously reported in the lung (12). SS at increased steady-state flow rates (HFR) also produced P_{pa} below those observed at the same flow rates during the generation of the SS pressure-flow curves, and this reduction was similar to that produced by the HVS (LFR) condition (Fig. 2*C*). This phenomenon was not observed in SS (LFR) lungs (Fig. 2*C*). Taken together, these

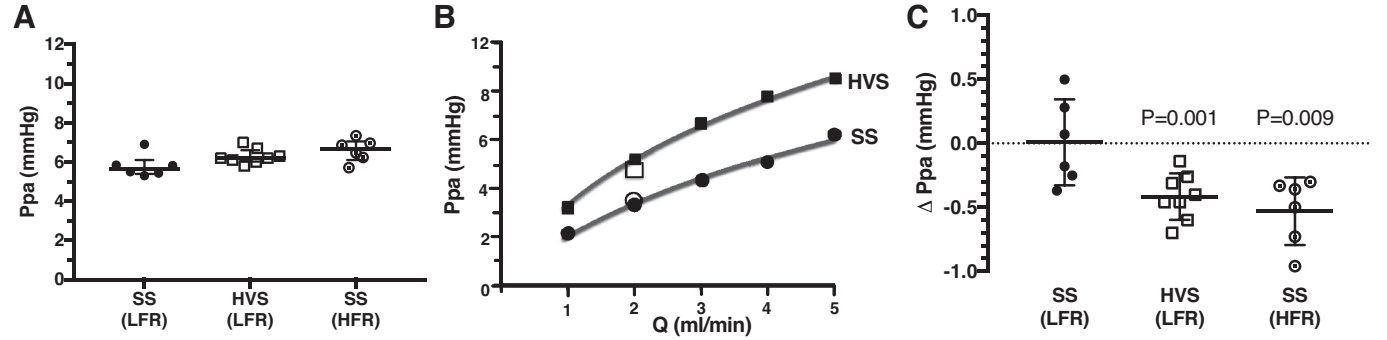

Fig. 2. *A*: median (IQR) pulmonary artery pressure (P_{pa}) during the steady state (i.e., before any increase in peak airway pressure) in each lung perfused with standard solution (SS) at a low flow rate (LFR), high-viscosity solution (HVS) at low flow rate (LFR), or SS at high flow rate (HFR). *B*: representative sample P-Q curve in a lung perfused with SS and in a lung perfused with HVS. Open symbol indicates the P_{pa} during the period of steady-state low flow perfusion following the initial collection of measurements for the P-Q curve. Open circle indicates value in the lung perfused with SS (LFR) and open square indicates the value in the lung perfused with HVS (LFR). *C*: median (interquartile range) difference (Δ) between the P_{pa} measured during steady-state flow in each lung in the 3 experimental groups and the corresponding P_{pa} determined from the P-Q curve for that lung. *P* values indicate statistical significance of the difference from zero (one group *t*-test).

findings suggest that increased shear rate, whether due to increased viscosity at constant flow rate or increased flow rate with unchanging viscosity, caused a similar reduction in pulmonary vascular resistance, compatible with shear-dependent vasodilatation.

In a further series of experiments, we examined P_{cap} during steady-state perfusion following initial perfusion at incrementing flow rates between 1.0 and 5.0 ml/min. HVS (LFR) did not cause a significant change in P_{cap} compared with SS (LFR) (Fig. 3*A*). This finding excluded the possibility that HVS (LFR) protected against edema by reducing hydrostatic filtration pressure within the capillaries and thus suggested that it reduced endothelial permeability. P_{pa} increased only to a small extent in the HVS (LFR) group, similarly to the previous series of experiments (Fig. 2*A*) and again compatible with a significant reduction in vascular resistance during HVS (LFR) perfusion. In keeping with this, transpulmonary pressure gradient (P_{tpg}) increased by a similar margin (Fig. 3*C*); the proportion of total vascular resistance in the arterial segment (R_a/R_t) was not significantly changed (Fig. 3*D*).

In contrast, the hemodynamic effects of SS-HFR were markedly different from those of HVS (LFR) even though both caused a similar increment in shear stress. SS (LFR) caused a significant increase in P_{cap}, P_{pa}, and P_{tpg} while leaving R_a/R_t unchanged compared with HVS (LFR) (Fig. 3). All of these changes are compatible with the higher vascular resistance R_0 (H_2O) seen in SS (HFR) conditions compared with HVS (LFR) and suggest that increased filtration pressure in the capillaries contributed to a more rapid formation of edema in this group (Fig. 1*C*).

Endothelial barrier function in the intact lung is improved by perfusion with high-viscosity solution. In further, independent experiments, we examined the effect of HVS on endothelial barrier function during extended steady-state perfusion. Each SS (LFR) perfused lung preparation was perfused until edema developed (median period 114 min, IQR 70–128), and a matched lung was then perfused with HVS (LFR) at the same low flow rate for an identical interval.

Mean wet-to-dry weight ratio was significantly higher ($P < 0.001$) in the SS-perfused lungs than in the HVS-perfused lungs, confirming the presence of edema (Fig. 4*A*). Extravasation of Evans Blue-labeled albumin was reduced by approximately one-half ($P < 0.001$) in the HVS-perfused lungs compared with that in SS (LFR) lungs (Fig. 4*B*), demonstrating that perfusion with HVS (LFR) reduced endothelial barrier permeability.

Effect of shear stress on the pulmonary endothelium in vitro. To gain further insight into the reduced endothelial permeability caused by HVS in the intact lung, we examined its effects on pulmonary arterial endothelial monolayers in vitro. We first compared the effect on endothelial barrier function of lower and higher shear stresses produced by altering viscosity without changing flow. Pulmonary endothelial monolayers were established by growing cells in HVS at a shear stress in the physiological range (10 dyn/cm^2). Monolayers were then switched to one of three different conditions for a further 4 h: reduced shear stress produced by the lower viscosity SS (7.0 dyn/cm^2), a higher shear stress produced by HVS (10 dyn/cm^2), or SS to which high-molecular-mass dextran (500 kDa) was added (HVS Dextran) to produce a relative viscosity (2.5) and shear stress (10 dyn/cm^2) identical to that of HVS. The low-shear stress condition (SS) increased the permeability of the monolayer formed by the cells when compared with HVS Ficoll and compared with HVS Dextran (Fig. 5*A*). The shear stress-dependent phosphorylation of eNOS was higher in both high viscosity solutions, confirming similar, shear stress-induced actions (Fig. 5*B*). Furthermore, occludin phosphotyrosine residues were reduced when the high-viscosity solutions

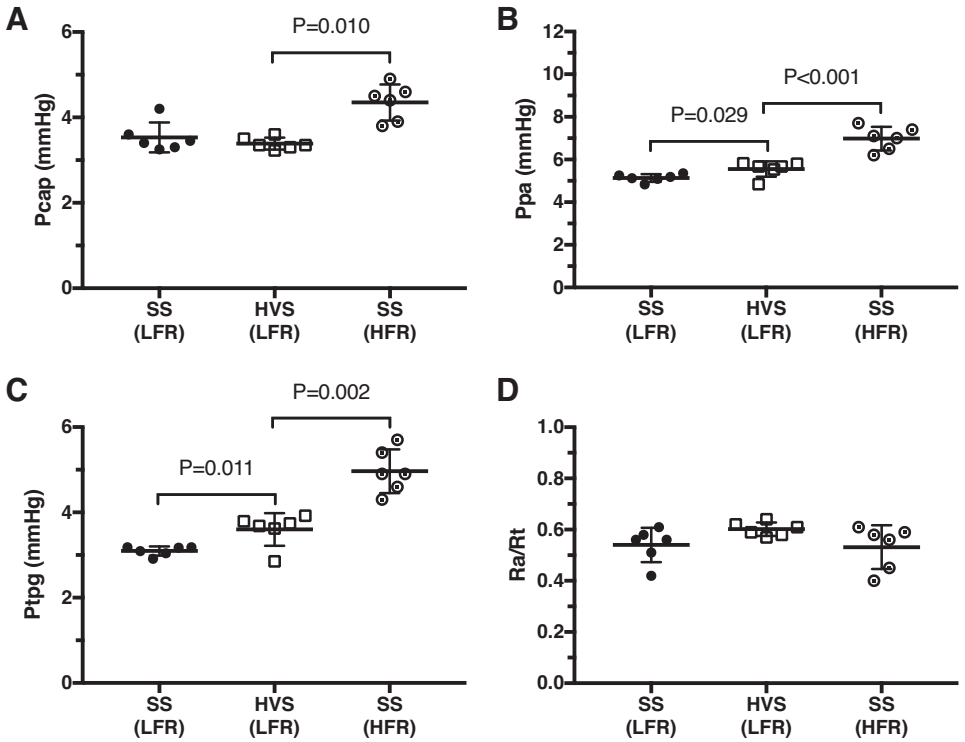

Fig. 3. *A*: mean (SD) capillary pressure (P_{cap}) in groups of lungs during steady-state perfusion with standard solution (SS) at low flow rate (LFR), with high-viscosity solution (HVS) at low flow rate (LFR), and with SS at high flow rate (HFR). Mean (SD) pulmonary artery pressure (P_{pa}) (*B*), mean (SD) transpulmonary pressure gradient (P_{tpg}) (*C*), and mean (SD) ratio of arterial resistance (R_a) to total resistance (R_t) measured at the same time (*D*). *P* values indicate statistical significance of the difference between groups (unpaired *t*-test with Holm-Sidak correction).

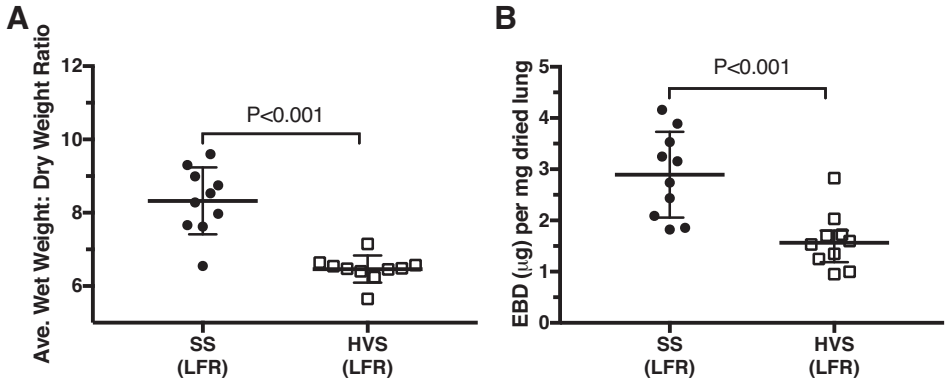

Fig. 4. Mean (SD) wet:dry weight ratio (*A*) and Evans blue dye (EBD) labeled albumin accumulation (*B*) in ex vivo lungs perfused with standard solution (SS) at low flow rate (LFR) and lungs perfused with high-viscosity perfusate (HVS) at the same low flow (LFR) rate for matched durations. *P* values indicate statistical significance of the difference between groups (unpaired *t*-test).

were used (Fig. 5*E*), a known shear stress-dependent phosphorylation reaction that stabilizes occludin, thus maintaining tight junction integrity and reducing endothelial permeability (36). Taken together these data show that higher shear stress caused improved barrier function of pulmonary endothelium in vitro, in agreement with previous reports in endothelial monolayers (15, 36).

We next compared the effects of higher shear stress (10 dyn/cm^2) produced in two different ways: HVS Ficoll at a lower flow rate or SS at a higher flow rate. Shear stress induced by both methods had identical effects on endothelial permeability, eNOS phosphorylation, and occludin phosphorylation (Fig. 5, *B*, *D*, and *F*). Thus the effect of higher shear stress to improve endothelial barrier function in vitro was identical whether produced by increased viscosity or increased flow.

DISCUSSION

The central roles of the hydrostatic pressure gradient, the oncotic pressure gradient, and the selective permeability of the endothelial barrier in the maintenance of normal fluid flux across the microvasculature have long been recognized. Our results show for the first time that, in the intact lung, increasing the viscosity of the perfusion solution above that of plasma but within the range observed in normal blood in vivo reduced fluid filtration in the intact pulmonary microvasculature and prevented edema formation by improving endothelial barrier function. Although the effect of increased viscosity in reducing vascular resistance through shear stress-induced vasodilatation is well described (9, 12), to our knowledge, the important role of the viscosity of the perfusion solution in regulating endothelial barrier function and fluid exchange has not previously been reported.

The important novel finding that we report is that increasing the viscosity of a solution perfusing the pulmonary circulation above that of normal plasma reduces vascular leak and thus reduces the rate of edema formation. We tested three possible mechanisms by which HVS (LFR) might have reduced edema formation: increased osmotic pressure of the HVS, reduced filtration pressure (P_{cap}), or reduction of endothelial permeability. We found that HVS did not act through the small increase in osmotic pressure that it caused, since a solution that had a similarly increased osmotic pressure but a much smaller increase in viscosity (6% Ficoll, Table 1) did not extend the time to edema; nor did HVS act by reducing the hydrostatic filtration pressure since it did not reduce P_{cap} compared with SS (LFR) (Fig. 3). The major mechanism that prevented an increase in P_{cap} was the HVS-induced reduction in vascular hindrance (R_0H_2O), which prevented a much larger increment in P_{pa} and P_{tpg} than would otherwise have occurred (see *Eq. 6*). Thus HVS prevented edema formation by improving endothelial barrier function as shown by the reduction in permeability to albumin that it produced (Fig. 4).

Interestingly, increasing endothelial shear stress in the intact pulmonary circulation by a different mechanism, i.e., by increasing flow rate without change in viscosity, did not have the same beneficial effect on the rate of edema formation and therefore the reduced edema formation cannot be explained by the effect of increased shear stress alone (Fig. 1*C*). Furthermore, we show that the mechanism of the effect in the intact circulation could not be reproduced in cultured endothelial cell monolayers (Fig. 5). This is clearly illustrated by the very different actions of the two different methods of increasing shear in the intact organ and in the pulmonary endothelial monolayers. Increased shear stress cause by increasing solution viscosity (at constant flow) reduced endothelial permeability both in pulmonary endothelial monolayers in vitro and in the intact lung (Fig. 5*B*). Increased shear stress produced by increasing flow at constant low viscosity, SS (HFR), also reduced endothelial permeability in the isolated monolayer (Fig. 4, *A* and *B*), as effectively as HVS at low flow rate (Fig. 5). In support of this, both methods of increasing shear stress in vitro caused similar increases in eNOS phosphorylation and similar reductions in occludin phosphorylation (Fig. 5), actions compatible with a shear-dependent tightening of endothelial junctions and improved barrier function, as previously reported in systemic endothelial monolayers (15, 36). In contrast, increased flow rate in vivo did not reduce edema formation (Fig. 1). Interestingly, this finding is compatible with previous reports in isolated vessels showing that increased flow is either without effect on vascular permeability, or increases it (1, 21). Taken together with these previous reports, our data suggest that HVS (LFR) did not protect against edema formation by a direct action of shear stress on the vascular endothelium.

Since high flow of the standard solution had very different effects on endothelial permeability in vivo and in vitro, we considered the possibility that the pulmonary arterial endothelial cells that we used (BPAECs) responded to shear stress in a way that differed from pulmonary microvascular endothelial cells. It is well known that endothelial cells from different organs and different regions within the vascular tree are heterogeneous in some aspects of their phenotype (2, 3). For example, it has previously been reported that cerebral micro-

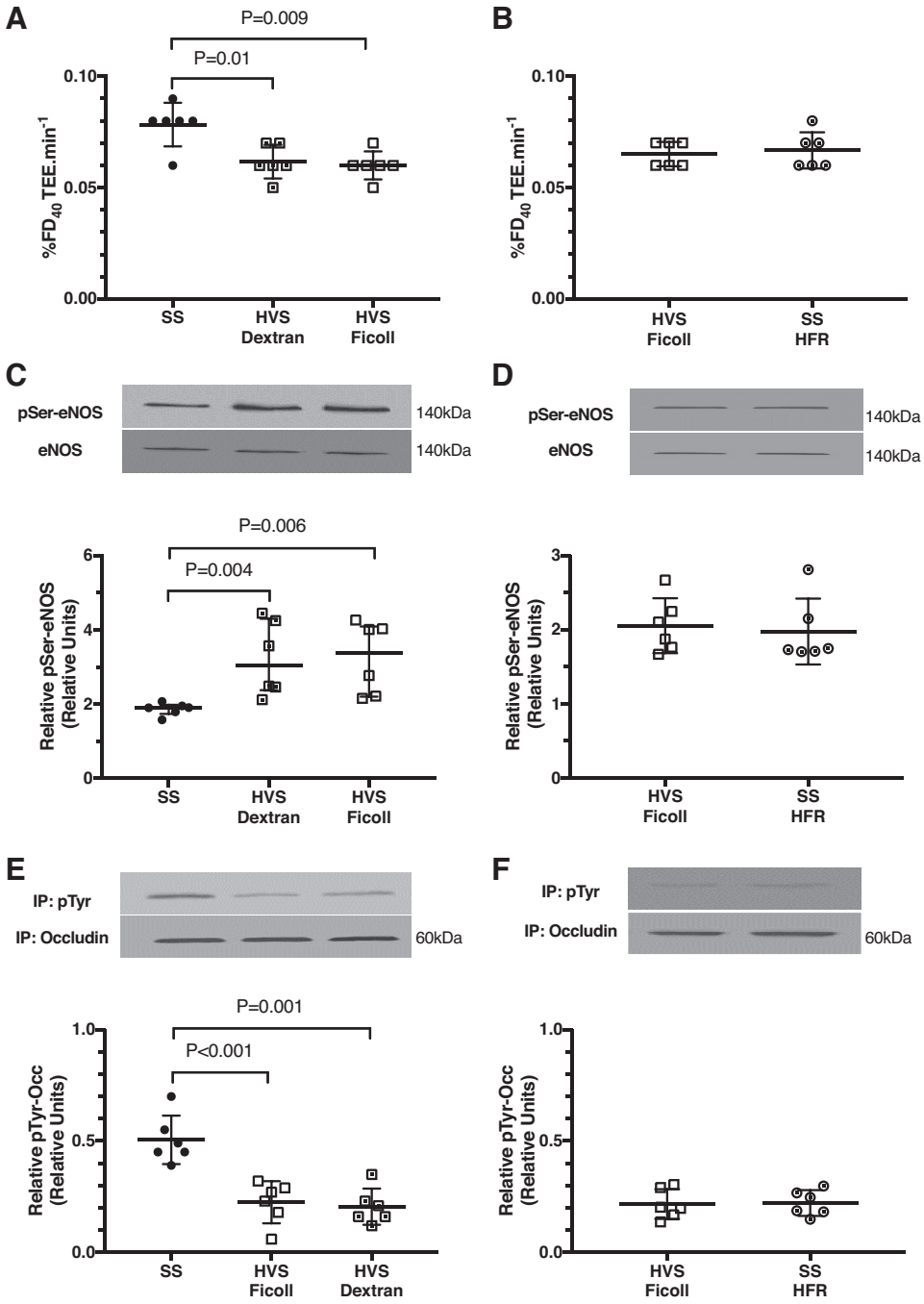

Fig. 5. *A*: mean (SD) rate of FITC-labeled dextran diffusion [%FD_{40} transendothelial exchange (TEE)/min] across endothelial monolayers cultured at the same flow rate in SS, and two different high-viscosity solutions, HVS Dextran solution (relative viscosity 2.5) and the HVS Ficoll solution (relative viscosity 2.5). *B*: mean (SD) rate of FITC-labeled dextran diffusion (%FD_{40} TEE/min) across endothelial monolayers cultured in HVS Ficoll solution at the same flow as in *A*, and SS at a higher flow rate so that the shear stress was the same as HVS Ficoll. *C* and *D*: representative Western blots and mean (SD) densitometry values for endothelial nitric oxide synthase (eNOS) serine (pSer) phosphorylation expressed relative to total eNOS under the same experimental conditions as *A* and *B*, respectively. *E* and *F*: representative Western blots and mean (SD) pTyr-Occ expressed relative to total occludin under the same experimental conditions as *A* and *B*, respectively. *P* values indicate statistical significance of the difference between groups (*t*-test with Holm-Sidak correction).

vascular endothelial cells do not show flow alignment in contrast to larger-vessel endothelial cells (38). However, in separate experiments we have found that human microvascular lung endothelial cells (HMvLECs) in vitro showed flow alignment identical to that seen in BPAECs and that occludin phosphorylation was inhibited in HMvLECs to the same extent in response to both HVS-LFR and SS-HFR (unpublished data), i.e., a response identical to that which we observed in BPAECs (Fig. 5, *E* and *F*). Others have also reported flow alignment in pulmonary microvascular endothelial cells (19). Nonetheless, although these results demonstrate that BPAECs recapitulated key features of pulmonary microvascular endothelial cells that are directly relevant to our experiments, it is important to remember that they may differ phenotypically in other important ways that we did not investigate. Additionally, the in vitro environment of the endothelial cells is very different from their microenvironment in vivo and this may have caused some important differences in phenotypic features from those they display in the intact pulmonary circulation (4, 34). This possibility is supported by previous reports showing that in isolated vessels, where the vascular endothelium remains in its normal environment, increased flow is either without effect on vascular permeability, or increases it (1, 21).

Since the increased shear stress induced by HVS (LFR) cannot account for the barrier-protective effects that it exerted in the intact lung, alternative mechanisms for the reduced

edema formation observed in HVS (LFR) conditions must be considered. One potential explanation for the protective action of HFS (LFR) is that it caused flow to be more homogeneously distributed than SS at the same low flow rate, SS (LFR). Heterogeneous perfusion in the lung causes overperfusion pulmonary edema, as seen in high-altitude pulmonary edema or multiple microemboli (31). In these conditions, reduction of flow to some areas of the lung (due to microemboli or greater hypoxic vasoconstriction) causes excessive flow and shear stress in the other regions of the lung, which leads to endothelial leak and edema formation. This possibility of more homogeneous distribution of flow in HVS (LFR) perfusion is supported by our observation of slower, patchy clearance of blood in SS-perfused lungs compared with a more homogeneous, rapid, and complete clearance of blood from HVS-perfused lungs. The markedly reduced R_0H_2O and absence of increase in P_{cap} in HVS (LFR) that we observed is also compatible with recruitment and redistribution of flow. Further experimental work is needed to test these potential explanations.

The novel finding that viscosity is a physiological property of a perfusion solution that regulates endothelial permeability and fluid balance in the microcirculation has important implications for our understanding of the physiological maintenance of fluid balance in tissues in vivo. Viscosity is not one of the physicochemical properties considered in the classic Starling hypothesis. Our findings also have important implications for understanding the pathophysiology of edema formation in disease. For example, severe anemia causes widespread edema that cannot be explained by increased capillary filtration pressures (5). Our data suggest that the reduced viscosity of profoundly anemic blood may be an important factor (14). Another clinical circumstance in which altered viscosity may contribute to edema formation is where acellular solutions have to be used for emergency fluid replacement therapy and support during resuscitation of critically ill patients. A further consequence of our findings is that viscosity should be considered in the design of artificial perfusion solutions used in experimental isolated organ preparations and in ex vivo lung perfusion to recondition donor organs for transplant programs (7, 16, 28).

We do not mean to suggest that viscosity is the only property of blood that accounts for the extended time to edema formation observed in blood-perfused lungs when compared with artificial perfusates, nor do we mean to suggest that the high-viscosity solution that we used mimics exactly the rheological properties of blood. Clearly this is not the case since the viscosity of SS and HVS was independent of shear rate in contrast to blood, the viscosity of which is shear rate dependent. Nonetheless, our data show that the viscosity of a perfusion solution has an important impact on endothelial permeability and edema formation in the intact circulation and suggest that viscosity is one of the important properties of blood that contributes to the maintenance of normal fluid balance within the microcirculation in vivo.

In summary, we have shown for the first time that the viscosity of the solution perfusing the pulmonary vascular bed is a previously unrecognized, physiological property that is centrally important for normal endothelial barrier function and the maintenance of normal fluid exchange in the intact microcirculation. These findings have important implications for our understanding of tissue fluid balance in health, and edema formation in disease.

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DISCLOSURES

S. C. Rowan and P. McLoughlin are named as inventors on a patent application by University College Dublin, entitled "A lung perfusion solution and use thereof for the ex vivo preservation of a mammalian lung."

AUTHOR CONTRIBUTIONS

S.C.R. and P.McL. conceived and designed research; S.C.R., K.D.R., and L.P. performed experiments; S.C.R., K.D.R., L.P., and P.McL. analyzed data; S.C.R., K.D.R., L.P., P.M.C., M.O'R., and P.McL. interpreted results of experiments; S.C.R. and K.D.R. prepared figures; S.C.R. and P.McL. drafted manuscript; S.C.R., K.D.R., L.P., P.M.C., M.O'R., and P.McL. edited and revised manuscript; S.C.R., K.D.R., L.P., P.M.C., M.O'R., and P.McL. approved final version of manuscript.

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