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Cytoskeletal remodeling and cellular activation during deformation of neutrophils into narrow channels

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Yap, Belinda, and Roger D. Kamm. Cytoskeletal remodeling and cellular activation during deformation of neutrophils into narrow channels. *J Appl Physiol* 99: 2323–2330, 2005. First published August 25, 2005; doi:10.1152/jappphysiol.00503.2005.—Neutrophils are subjected to mechanical stimulation as they deform into the narrow capillary segments of the pulmonary microcirculation. The present study seeks to understand the changes in the cytoskeletal structure and the extent of biological activation as a result of this process. Neutrophils were passed through narrow polycarbonate filter pores under physiological driving pressures, fixed, and stained downstream to visualize the F-actin content and distribution. Below a threshold capillary size, the cell remodeled its cytoskeleton through initial F-actin depolymerization, followed by recovery and increase in F-actin content associated with formation of pseudopods. This rapid depolymerization and subsequent recovery of F-actin was consistent with our previous observation of an immediate reduction in moduli with eventual recovery when the cells were subjected to deformation. Results also show that neutrophils must be retained in their elongated shape for an extended period of time for pseudopod formation, suggesting that a combination of low driving pressures and small capillary diameters promotes cellular activation. These observations show that mechanical deformation of neutrophils into narrow pulmonary capillaries have the ability to influence cytoskeletal structure, the degree of cellular activation, and migrational tendencies of the cells.

F-actin; pseudopod; migration; pulmonary capillaries

AS NEUTROPHILS TRAVEL FROM the arterial to the venous side of the pulmonary microcirculation, they must traverse 50–100 capillary segments (11, 13, 14), ranging in diameter from 2 to 15 μm (5). When neutrophils encounter a capillary segment narrower than their size (6–8 μm), they will have to deform to pass through.

Early studies on the behavior of neutrophils during deformation into narrow capillaries assumed that the cell remained passive throughout the process (9, 23, 27, 28) and that the induced stresses had no effect on cell behavior. However, recent studies have begun to address the dynamic response of leukocytes as a result of mechanical stimulation. In particular, mechanical deformation was shown to upregulate adhesion molecules, reorganize and stabilize the cell cytoskeleton, and increase free intracellular Ca^{2+} concentration (19). Neutrophils have also been shown to respond to fluid shear stress by reducing their internal stiffness, altering their adhesion to substrates, as well as generating pseudopod projections (4, 20, 22). In our recent study (31), we designed an *in vitro* microchannel and associated flow system to study the response of

neutrophils to mechanical deformation, mimicking the physiological conditions experienced during passage through pulmonary microvessels. Results from rheological studies showed an immediate reduction in the complex shear modulus, with subsequent recovery within 1 min, suggesting that the neutrophil rapidly remodeled its cytoskeleton when subjected to mechanical deformation. Above a threshold stimulus, mechanical stimulation resulted in cell activation evidenced by projection of pseudopods. In the present work, we investigate the mechanism(s) governing this cytoskeletal remodeling process that takes place when the cell undergoes mechanical deformation.

MATERIALS AND METHODS

Preparation of human neutrophils. Human neutrophils were isolated from healthy adult volunteers by Histopaque centrifugation, dextran sedimentation, followed by hypotonic lysis of contaminating erythrocytes, as described previously (31). The isolated neutrophils were suspended in medium [Hanks balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+} supplemented with 2% autologous plasma]. The cells were counted, and the concentration was adjusted to 5.0×10^5 cells/ml. Subsequently, the neutrophils were kept either at room temperature or incubated in a 37°C water bath according to the required experimental conditions.

Constant-pressure *in vitro* filtration system. The constant-pressure filtration system consisted of a small reservoir connected to a filter holder on the downstream end and to a large reservoir on the upstream side (Fig. 1). Both the small reservoir and the filter holder were enclosed in water jackets and maintained at constant temperature (either 23 or 37°C). Two different polycarbonate filters were used (GE Osmonics, Minnetonka, MN): one with a pore diameter of 3 μm , pore length of 9 μm , and pore density of $2.0 \times 10^6/\text{cm}^2$; and another with a pore diameter of 5 μm , pore length of 10 μm , and pore density of $4.0 \times 10^5/\text{cm}^2$. To minimize adhesion of neutrophils (17), before the start of experiments, the polycarbonate filters were immersed in a 1% Pluronic F108 solution (PEO/PPO/PEO triblock copolymers, BASF, Mount Olive, NJ) in water for 2 h and subsequently flushed with medium for 15 min.

Medium was first introduced throughout the system with syringes 1 and 2 (Fig. 1) after equilibrating with the constant-temperature water jackets for at least 10 min. Neutrophils were then introduced via syringe 1 into the small reservoir, ready for the filtration process. Pressure upstream of the filter could be changed by adjusting the height of the reservoir relative to the height of the filter with a linear vertical slide (Rapid Advance Unislides, Velmex, Bloomfield, NY). Finally, the valve was set to allow the neutrophils to flow directly into the filter holder. With the use of the large reservoir (to ensure that the level of fluid in the reservoir remained nearly constant during the experiment), neutrophils were passed through the filter holder at a

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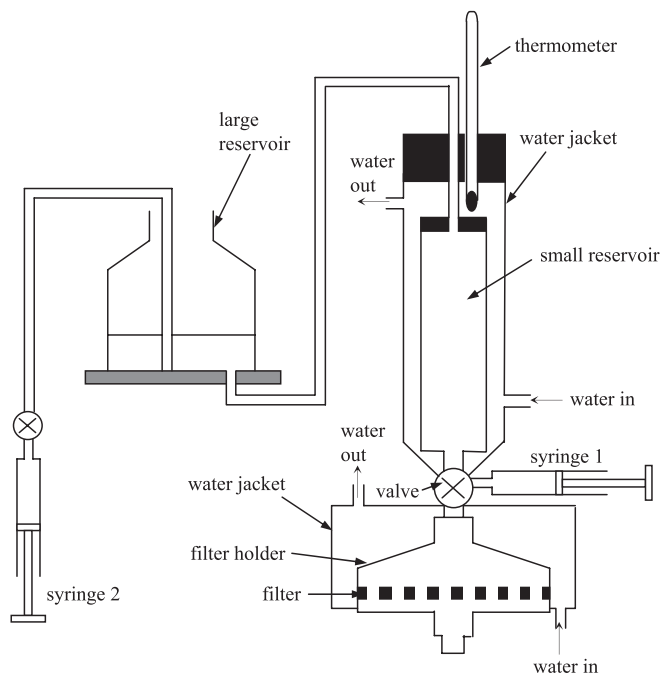


Fig. 1. Schematic showing design of the constant-pressure *in vitro* filtration setup. Neutrophils flowed through the filters at a rate determined by the height of the large reservoir. Postfiltered cells were subsequently collected and fixed in paraformaldehyde. Refer to MATERIALS AND METHODS for further description.

constant pressure difference, which for this study was either 5 or 19.5 cmH₂O. The average volume flow rate of filtration was obtained by dividing the filtrate volume by the collection time.

Constant flow rate *in vitro* filtration system. The system for constant-flow rate filtration is designed after one used previously (19, 21). Neutrophils at concentration of 5.0×10^5 cells/ml were loaded into a 12-ml polypropylene syringe (Monoject, Sherwood Davis & Geck, St. Louis, MO) that was mounted onto a syringe pump (pump 22, Harvard Apparatus, Holliston, MA) capable of delivering the sample fluid at a constant flow rate. Neutrophils were filtered through polycarbonate filters at three different flow rates (1.0, 3.0, and 6.0 ml/min) at room temperature ($\sim 23^\circ\text{C}$). The filter used has a pore diameter of 3 μm , with pore length and density as specified above. As before, the filters were also treated with 1% Pluronic F108 solution before use. Pressure upstream of the filter was continuously monitored with a pressure transducer (Validyne Engineering, Northridge, CA).

Quantitation of neutrophil morphology. Neutrophils filtered through the polycarbonate filters were fixed immediately in paraformaldehyde (4% vol/vol) for 20 min at room temperature and washed twice in HBSS. For each preparation ($n = 3$), 50 neutrophils were chosen randomly, and the cells were observed by light microscopy (Eclipse TE300, $\times 60$, Melville, NY). Images were analyzed to determine cell circularity using the image analysis software ImageJ version 1.33 (National Institutes of Health, Bethesda, MD). Circularity [$4\pi \times \text{area} / \text{perimeter}^2$] of the cell approaches 1.0 for a perfect circle; lower values reflect a progressively elongated ellipse.

Quantification of F-actin content. As above, resting neutrophils or neutrophils filtered through the polycarbonate filters were fixed immediately in paraformaldehyde (4% vol/vol) for 20 min at room temperature and washed twice in HBSS. Cells were then incubated in a final concentration of 2 μM lysophosphatidylcholine (Sigma-Aldrich, St. Louis, MO) and 0.165 μM tetramethylrhodamine isothiocyanate-conjugated phalloidin (Sigma-Aldrich) for 30 min in the dark at 37°C for permeabilization and staining, then washed and resuspended in HBSS. Stained cells were filtered (35- μm mesh nylon filter), and the F-actin content was analyzed using a flow cytometer

(Cytomics FC500, Beckman Coulter, Fullerton, CA) within 1 h of staining. A total of 20,000 cells were analyzed for each preparation ($n = 3$). In all cases, the fluorescence histogram was a normal distribution from which the fluorescence mean was determined. The relative F-actin content of filtered neutrophils is the ratio of the mean intensity of the filtered samples to the mean intensity of resting neutrophils at the corresponding temperature (23 or 37°C). As a positive control, neutrophils were stimulated with 10^{-7} M *N*-formyl-methionyl-leucyl-phenylalanine (Sigma-Aldrich) for 10 s. In the case of neutrophil filtration through 3- μm filters at 37°C , the neutrophils were also fixed at 15, 30, 60, and 120 s postfiltration and the corresponding F-actin content was quantified.

Visualization of F-actin reorganization and analysis of polarization in neutrophils. Neutrophils prepared as described above for flow cytometry analysis was visualized under a fluorescent microscope (Eclipse TE300, $\times 60$, Melville, NY) to determine the distribution of F-actin. For each preparation ($n = 3$), 50 cells were chosen randomly, and neutrophils showing asymmetrical distribution of F-actin were scored as morphologically polarized. The percentage of polarized neutrophils was computed for each experimental condition.

Statistical analysis. All results are expressed as means \pm SE. Data comparisons for circularity and F-actin content were carried out using the Student's *t*-test, whereas comparisons of results for polarity were analyzed using χ^2 test. Findings that showed either $P < 0.05$ or $P < 0.01$ were considered significant.

RESULTS

Neutrophils were passed through polycarbonate filters using two different filtration setups: constant pressure and constant flow rate. For the constant-pressure system, measurements of the average volume flow rates are shown in Table 1. Upstream pressures attained a nearly steady state after 1 min of filtration for the constant-flow rate system, the values of which are reported in Table 1.

Shape of neutrophils that undergo large-scale deformations is affected by the driving force. Cells that had deformed through the polycarbonate filters were fixed immediately, and the shape departure from circular was quantified. Figure 2A shows the circularity index of neutrophils deformed under constant pressure. In 3- μm -filtered cells, an increase in filtration pressure from 5 to 19.5 cmH₂O resulted in a significant increase ($P < 0.01$) in elongation of the neutrophils, at both temperatures of 23 and 37°C . In contrast, comparison between 5- μm -filtered cells showed that filtration pressure has no effect on the circularity index. The circularity index for neutrophils deformed under constant flow rate is depicted in Fig. 2B.

Table 1. Pressures and corresponding flow rates for the *in vitro* filtration systems

Experimental Conditions	23°C		37°C	
	3- μm filtration	5- μm filtration	3- μm filtration	5- μm filtration
Constant-pressure system, ml/min				
19.5 cmH ₂ O	3.5 \pm 0.6	64 \pm 4.0	5.4 \pm 0.9	74 \pm 9.0
5 cmH ₂ O	0.5 \pm 0.1	4.9 \pm 0.3	0.7 \pm 0.1	5.3 \pm 0.5
Constant-flow rate system, cmH ₂ O				
6 ml/min	28.4 \pm 4.8			
3 ml/min	20.6 \pm 3.0			
1 ml/min	6.2 \pm 0.9			

Values are means \pm SE.

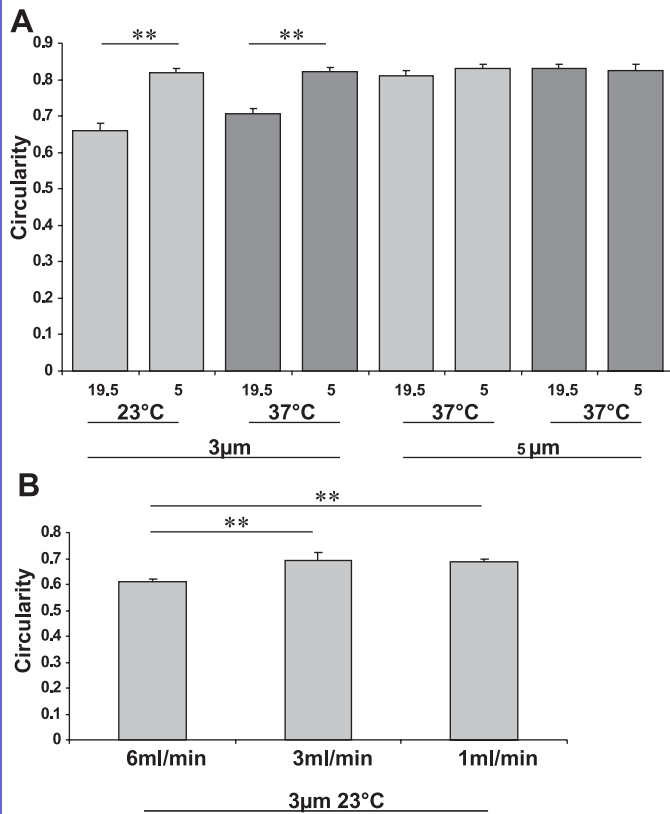


Fig. 2. A: circularity index of neutrophils deformed under constant pressure. The horizontal axis represents the different filtration pressures (in units of cmH₂O), temperatures, and filter pore sizes. B: circularity index of neutrophils deformed under constant flow rate. The horizontal axis represents the different filtration flow rates, temperatures, and filter pore sizes. Values are means \pm SE ($n = 3$). $**P < 0.01$.

Comparison of results for 3- μ m-filtered cells at 23°C showed a significant elongation ($P < 0.01$) between cells filtered at 1 and 6 ml/min, and between cells filtered at 3 and 6 ml/min. However, the increase in filtration flow rate from 1 to 3 ml/min did not change the neutrophil circularity index. These findings suggest that neutrophil shape is affected by driving pressure for cells experiencing large-scale (3- μ m pores) but not small-scale (5- μ m pores) deformations.

Neutrophils exhibit a rapid fall and recovery in F-actin content and distribution after large-scale deformation. Measurements of F-actin content for neutrophils deformed under constant pressure and constant flow rate, respectively, and fixed immediately after filtration are shown in Fig. 3, A and B. Neutrophils passing through 3- μ m filters under a constant pressure of 19.5 cmH₂O showed a significant decrease in their F-actin content (Fig. 3A). In contrast, when the cells were filtered at 5 cmH₂O, they displayed a significant increase in F-actin content. Cells deformed through 5- μ m filters under both pressures did not show any changes in their fluorescence intensity compared with resting values. Stimulation with 10^{-7} M *N*-formyl-methionyl-leucyl-phenylalanine for 10 s resulted in increased fluorescence intensity. This observation holds true for experiments conducted at 23 and 37°C. For the constant-flow rate system (Fig. 3B), experiments carried out for 3- μ m-filtered cells at 23°C showed a decrease in F-actin content for neutrophils filtered at 6 ml/min. However, cells filtered at 1 and

3 ml/min showed an increase in fluorescence intensity compared with resting cells. The pattern of changes in F-actin content are therefore quite complex, with no change occurring under small-scale deformation (5- μ m pores), whereas those undergoing large-scale deformation (3- μ m pores) exhibit either a decrease or increase in F-actin for high and low driving pressures, respectively.

Fluorescence microscopy revealed a diffuse distribution of F-actin for resting neutrophils at both 23 and 37°C (Fig. 4, A and B). Filtration through 3- μ m filters under a constant-pressure of 19.5 cmH₂O resulted in a redistribution of F-actin, forming a discontinuous border around the cell edges (Fig. 4C). In contrast, neutrophils deformed through 3- μ m filters under a lower constant pressure of 5 cmH₂O showed increased ruffling of the plasma membrane and formation of pseudopods, which were also sites of F-actin localization (Fig. 4D). However, filtration through 5- μ m filters under high or low constant pressure did not result in F-actin reorganization (Fig. 4E). In the constant-flow rate system, neutrophils passed through 3- μ m filters under a high flow rate of 6 ml/min exhibited F-actin localized discontinuously along the cell periphery (Fig. 4F). In contrast, cells filtered at flow rates of 1 or 3 ml/min showed a more uniform distribution of F-actin within the cellular interior (Fig. 4G), with occasional cells forming pseudopods (Fig. 4H).

Combination of small pore dimension and low driving force leads to neutrophil activation. Cells filtered through 3- μ m filters under constant-pressure showed significantly increased polarization ($P < 0.01$) when the pressure was reduced from 19.5 to 5 cmH₂O (Table 2). This observation holds true at both 23 and 37°C. In contrast, none of the neutrophils filtered through 5- μ m filters either at low or high pressures was polarized. Neutrophils deformed through 3- μ m filters under constant flow rate also showed a significant increase in polarization when the flow rate was decreased from 6 to 1 ml/min. No significant change in polarization was observed when comparing results for 6 and 3 ml/min or from comparison of the data for 3 and 1 ml/min.

Neutrophil allowed to recover after high driving pressure and large-scale deformation exhibit recovery in F-actin content and distribution. Neutrophils passed through 3- μ m filters under a constant pressure of 19.5 cmH₂O at 37°C, and subsequently fixed at different time points, showed a recovery of F-actin content to within 95% of baseline resting values (Fig. 5). The distributions of F-actin at different time points postfiltration are shown in Fig. 6. Neutrophils fixed 15 s after filtration still showed F-actin localization around the periphery of the cell. Compared with cells fixed at earlier time points, however, neutrophils fixed at 30 s and thereafter showed a recovery of F-actin in the cell interior.

DISCUSSION

Neutrophils often encounter narrow capillary segments during their passage through the pulmonary microcirculation, and as the cells deform to pass through these narrower segments they are subjected to mechanical stimulation. In a previous study (31), we examined changes in the rheological behavior of neutrophils subjected to these mechanical forces. Our new results show that mechanical stimulus has the ability to remodel the neutrophil cytoskeleton as well as induce cell acti-

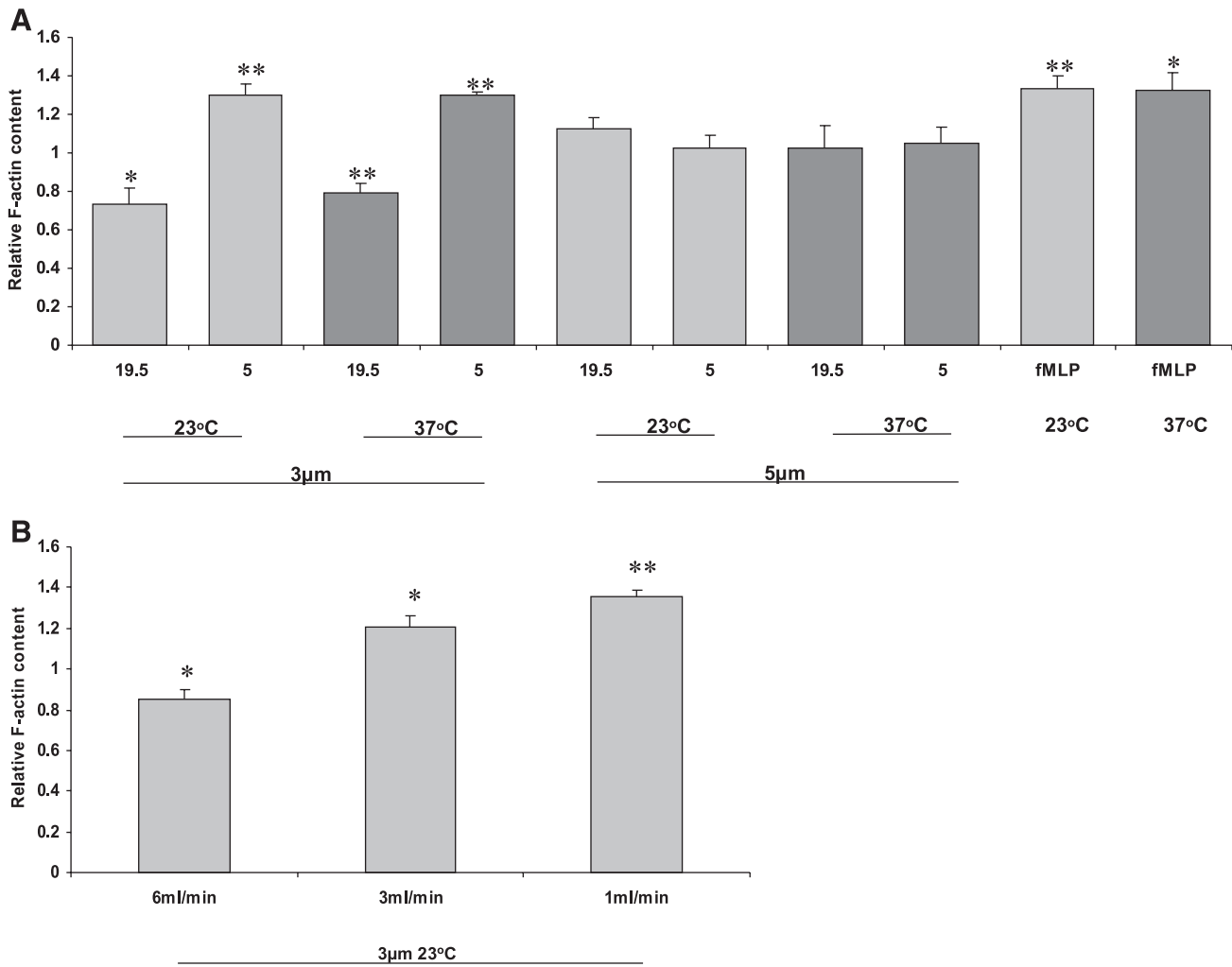


Fig. 3. A: relative F-actin content of neutrophils deformed under constant pressure. The horizontal axis represents the different filtration pressures (in units of cmH₂O), temperatures, and filter pore sizes. B: relative F-actin content of neutrophils deformed under constant flow rate. The horizontal axis represents the different filtration flow rates, temperatures, and filter pore sizes; *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) stimulated cells served as positive control. Values are means \pm SE ($n = 3$) and expressed as fraction of F-actin content in resting neutrophils. * $P < 0.05$; ** $P < 0.01$.

vation. Below a threshold pore size, mechanical deformation results in actin depolymerization and is followed later by cellular activation.

A constant-pressure filtration setup was designed to mimic the deformation of neutrophils in pulmonary capillaries, similar to that used in previous studies (1, 2, 7, 10, 24). In this system, the driving pressure across the filter was maintained constant over time by a pressure reservoir. Two different filter pore sizes were chosen, 3 and 5 μm diameters, which are typical diameters of the narrower pulmonary capillary segments. Neutrophils, which have average diameters of 6–8 μm (5), consequently must deform when passing through the polycarbonate filters. This setup allowed the neutrophils to be fixed and stained postfiltration, hence, permitting visualization of the actin cytoskeleton of the cells. Our previous study (31) utilized a microfluidic device, which was designed for single live cell imaging, allowing the determination of changes in shear modulus, but was not amenable to cell fixation and postfixation staining. In this new system, we employed a constant-pressure filtration system as opposed to a constant-flow rate system as used previously (6, 19, 25, 30), because

studies (12, 15) have shown that this is a better representation of the physiological condition experienced by neutrophils in the pulmonary microcirculation.

Using the constant-pressure system during neutrophil filtration, an increase in filtration pressure resulted in a corresponding increase in flow rate of the filtrate, as expected, with all other conditions (temperature and pore size) being equal (Table 1). Similarly, a larger pore size increased the flow rate of filtrate for a fixed filtration pressure and temperature. The effect of raising the experimental temperature from 23 to 37°C was to increase the filtrate flow rate, indicative of a more easily deformable cell at a higher temperature, as has been reported by others (8, 9, 16).

Increasing the driving pressure for 3- μm filtration from 5 to 19.5 cmH₂O produced a more elongated cell (Fig. 2A). In contrast, driving pressure had no observable effect on cells filtered through 5- μm pores. These results may be explained if we consider the time delay between deformation and fixation, which we term t_f . Due to the collection procedure, at low filtration flow rates, the elongated neutrophils postfiltration have the opportunity to partially recover their initial shape

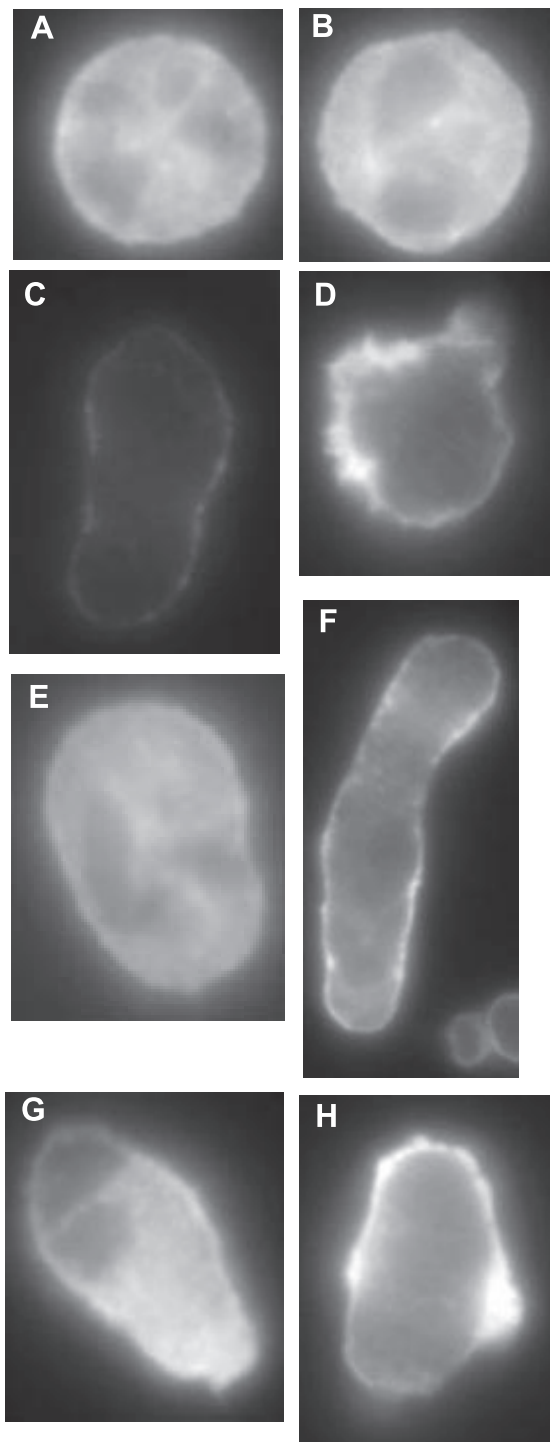


Fig. 4. Representative images showing distribution of F-actin and morphological changes in cells stained with tetramethylrhodamine isothiocyanate-phalloidin in resting neutrophils at 23°C (A), resting neutrophils at 37°C (B), neutrophils filtered through 3- μ m pores under constant pressure of 19.5 cmH₂O (C), neutrophils filtered through 3- μ m pores under constant pressure of 5 cmH₂O (D), neutrophils filtered through 5- μ m pores under constant pressure of 19.5 cmH₂O (similar F-actin distribution for 5 cmH₂O filtration; E), neutrophils filtered through 3- μ m pores under constant flow rate of 6 ml/min (F), and neutrophils filtered through 3- μ m pores under constant flow rate of 1 ml/min (similar F-actin distribution for 3 ml/min filtration; G and H).

before encountering fixative. Conversely, high filtration flow rates lead to rapid fixation, i.e., t_F is very short. Hence, in 3- μ m filtration, due to the low flow rate (Table 1) at 5-cmH₂O filtration pressure, neutrophils were less elongated at the time of fixation compared with cells that experienced the 19.5-cmH₂O filtration pressure. However, the larger pores of 5- μ m filters allowed high enough flows at both 5 and 19.5 cmH₂O that no differences were observed in the shape of the fixed cells.

In our previous study (31), we demonstrated that neutrophils subjected to mechanical deformation experienced an immediate decrease in both the storage and loss shear moduli. Within 1 min after stimulation, the shear moduli recovered to near initial values. Mechanical force also activated the neutrophils, as evidenced by the formation of pseudopods within 1 or 2 min of stimulation. In our present experiments, the neutrophils could be collected downstream after deformation, fixed, and their cytoskeleton revealed with appropriate staining. Hence, to understand the transient changes in neutrophil cytoskeleton as a result of mechanical deformation, the F-actin content of neutrophils was compared with resting cells. Cells filtered through 3- μ m pores under a constant pressure of 19.5 cmH₂O showed a decrease in F-actin content as measured by flow cytometry (Fig. 3A). In contrast, at a lower filtration pressure of 5 cmH₂O, the F-actin content was increased. This apparently anomalous behavior could be explained if we relate the present observations to results reported in Ref. 31. At higher filtration pressures, neutrophils were likely fixed at the initial stage of the remodeling process at a time when the shear moduli of the cells were reduced due to a combination of a short entrance/residence time in the filter under the high driving pressure. In contrast, at lower driving pressures, the neutrophils required more time to deform into the pores, so the cells had ample opportunity to form pseudopods before fixation. Previous studies have shown that neutrophils require \sim 2 s at 20-cmH₂O aspiration pressure to enter a 3- μ m pore, increasing to 15 s at 5 cmH₂O (12). Given that pseudopod formation occurs as early as 10 s after the onset of mechanical deformation (31), the neutrophils likely had ample time to form pseudopods for the 5 cmH₂O filtration while deforming into the pores. Experiments carried out previously in which neutrophils were deformed into microchannels under low pressure drop (close to threshold entrance pressure) (31) showed that neutrophils formed pseudopods in the microchannels while undergoing deformation (data not shown). The high F-actin content of pseudopods, therefore, may account for the increased F-actin content observed for filtration at lower driving force. This is

Table 2. Percentage of polarized cells after filtration

Experimental Conditions	23°C		37°C	
	3- μ m filtration	5- μ m filtration	3- μ m filtration	5- μ m filtration
Constant-pressure system				
19.5 cmH ₂ O	19 \ddagger	0	9 \ddagger	0
5 cmH ₂ O	69 \ddagger	0	67 \ddagger	0
Constant-flow rate system				
6 ml/min	11*			
3 ml/min	18			
1 ml/min	25*			

Significant difference between bracketed values: * $P < 0.05$; $\ddagger P < 0.01$.

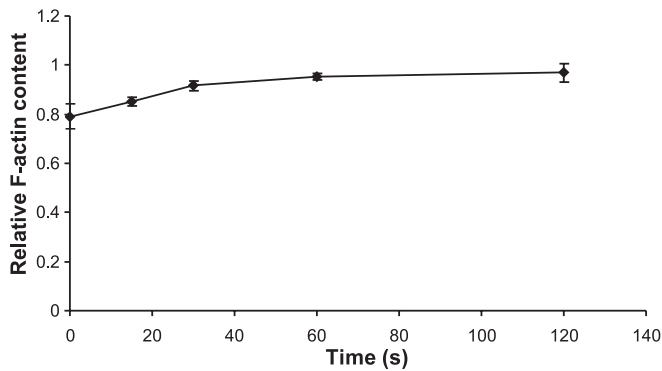


Fig. 5. Time course of F-actin content for neutrophils passed through 3- μ m filters under a constant pressure of 19.5 cmH₂O at 37°C.

also reflected in the results showing the percentage of polarized cells for each experimental condition (Table 2). Here, decreasing the filtration driving force clearly results in an increase in polarization of the neutrophil caused by formation of pseudopods. Pseudopod formation for the low driving pressure conditions can also be seen from F-actin stained images of the cells (Fig. 4D). In summary, it appears that neutrophils subjected to mechanical stimulus first undergo F-actin depolymerization, followed by recovery and a subsequent increase in F-actin content attributable to the formation of pseudopods.

To investigate the contribution of t_F to cell activation, neutrophils filtered through 3- μ m pores at high driving pressure were fixed at different time points after filtration. Figures 5 and 6 show that the F-actin content of the cells recover to their baseline resting value without causing cell activation, as reflected by pseudopod projection. Similar observations were noted in neutrophils flowing through the microchannel setup in Ref. 31. Neutrophils that were allowed to pass through the microchannels without trapping the cell recover to their original resting spherical shape without any evidence of pseudopod projection (data not shown). These results show that t_F has no effect on neutrophil activation. However, the results strongly suggest that, in addition to subjecting the neutrophils to mechanical stimulus through deformation into a narrower channel, the neutrophils must be retained in their deformed/elongated state for pseudopod to form. This implies that physiologically there are at least two ways in which neutrophils can form pseudopods in the pulmonary circulation: either if the entrance time of the cell exceeds the time to pseudopod projection (as mentioned above) or if the neutrophils become trapped in the capillaries, either due to the combined effects of low driving pressure and small capillary dimension or to active adhesion to the capillary endothelium.

A central question pertinent to cytoskeletal remodeling in neutrophils is the molecular mechanism governing the breakdown of the cytoskeleton due to mechanical deformation. Two hypotheses were presented in Ref. 31: sudden depolymerization of F-actin or rupture of actin cross-links between the filaments. The present experiments show that the initial rapid reduction in moduli is at least partially caused by depolymerization of F-actin. Further evidence of actin depolymerization during the early remodeling stage is provided by comparison of images of the cells before and after filtration. Neutrophils in the undeformed resting state exhibited a uniform distribution of F-actin over the entire cytoplasm (Fig. 4, A and B). However,

after filtration under high driving force, F-actin was concentrated around the periphery of the cells (Figs. 4C), indicative of an absence of F-actin in the central region of the cells due to depolymerization. However, we cannot rule out the possibility that the rupture of actin cross-links could also play a role in assisting this depolymerization process. In vitro results of Wirtz and colleagues (29) demonstrate that F-actin networks abruptly soften under high deformation at low concentrations of actin cross-linking proteins (filamin in this case), pointing to the role of actin-binding proteins in modulating cytoskeletal stiffness when subjected to mechanical deformation. In the case of neutrophils subjected to mechanical stimulus, both mechanisms, actin depolymerization and actin cross-link rupture, might occur simultaneously, a possibility being that the unbinding of the cross-links or even rupture of actin filaments themselves might initiate the depolymerization process (18).

Constant-pressure filtration of neutrophils through 5- μ m filters had no observable effect on the F-actin content of the cells (Fig. 3A). Postfiltered neutrophils also showed no evidence of polarization (Table 2) or reorganization of F-actin (Fig. 4E). Hence, these results suggest the existence of a threshold pore size below which the neutrophils sense the mechanical stimulus, remodel their cytoskeleton, and induce activation.

Although the constant-pressure setup is perhaps a better physiological model, the in vitro study of neutrophil deformation in pulmonary capillaries had previously been carried out under constant flow rate using a syringe pump to deliver the neutrophil solution through polycarbonate filters. Kitagawa et al. (19) investigated the role of mechanical deformation on the

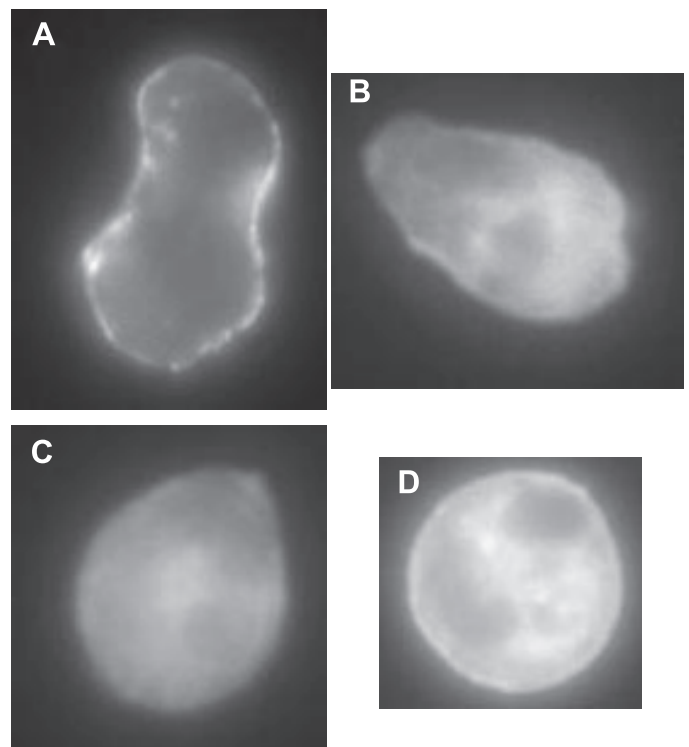


Fig. 6. Representative images showing distribution of F-actin in cells stained with tetramethylrhodamine isothiocyanate-phalloidin and fixed at 15 s (A), 30 s (B), 60 s (C), and 120 s (D) postfiltration. Image of cell fixed immediately after filtration (time = 0 s) is shown in Fig. 4C.

behavior of neutrophils under constant flow rate, and here we repeated and extended some aspects of their experiments to confirm our results and compare observations from the two systems. Using flow rates of 1 and 3 ml/min for 3- μ m filtration as in Ref. 19, we were able to reproduce their results and similarly show that postfiltered neutrophils at these flow rates attain comparable circularity index (Fig. 2B) as well as display an increase in F-actin content after filtration (Fig. 3B). However, since we did not observe the reduction in F-actin content of neutrophils, we suspected that at these low flow rates the cells were not fixed until reaching a later stage of the remodeling process. This suspicion was confirmed by experiments repeated at a higher flow rate of 6 ml/min, showing a significant reduction in the circularity index compared with 1 and 3 ml/min, indicative of a shorter t_F as discussed above (Fig. 2B). More importantly, there was indeed a reduction in F-actin content of the neutrophils (Fig. 3B), and depolymerization could also be observed from images of the cells (Fig. 4F). This confirmed our observation that neutrophils undergo an immediate but transient depolymerization of F-actin when subjected to mechanical deformation, which subsequently leads to formation of pseudopods. Nevertheless, although both the constant-pressure and constant-flow rate systems gave consistent results in this respect, there were discrepancies in other observations. From a comparison across the two setups (Table 1), it can be seen that the experimental conditions of 5 cmH₂O in the constant-pressure system is comparable to the 1 ml/min flow condition for the constant-flow rate system, and a similar matching occurs between the 19.5-cmH₂O constant-pressure system and the 3 ml/min constant-flow rate system. However, although we see a distinct difference in the response of neutrophils at 5 cmH₂O when compared with that at 19.5 cmH₂O, there was little difference in the behavior of the cells filtered at 1 and 3 ml/min. Only when the flow rate was stepped up to 6 ml/min did the neutrophils display responses similar to the higher filtration pressure of 19.5 cmH₂O. Furthermore, as shown in Table 2, neutrophils filtered under constant flow rate display less activation as a whole compared with constant-pressure filtration. Further tests are needed to reconcile the discrepancies observed in these two setups.

The scenario that emerges is one in which all cells subjected to a minimum level of elongation exhibit an initial drop in F-actin content due to depolymerization, followed by a recovery to initial values over a time scale of 30–60 s. This pattern accounts, at least in part, for the immediate reduction in shear moduli, with recovery over a time scale similar to that seen in previous experiments (31). Those cells that experience a prolonged period of deformation, however, as occurs in the combination of low driving pressures and small pore diameters, become activated. This activation is reflected in an eventual increase in F-actin content, over initial baseline values, accompanied by polarization and pseudopod formation.

These findings have important implications with respect to neutrophil transmigration in the pulmonary capillaries. Due to the highly interconnected nature of the alveolar microvascular bed (26), neutrophils will experience both a range of driving pressures and a range of capillary diameters, causing them to stop and undergo deformation to pass through. If the deformation is sufficiently large (capillary diameters of \sim 3 μ m) and the delay sufficiently long, the cells will become activated to

migrate. This would presumably be accompanied by increased expression of adhesion receptors (3, 19) and potentially lead to migration out of the capillary and into the extracellular matrix of the lung.

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