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Fluid Shear Attenuates Endothelial Pseudopodia Formation into the Capillary Lumen

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Abstract

Objective—Endothelial cells have the ability to undergo morphological shape changes, including projection of cytoplasmic pseudopodia into the capillary lumen. These cytoplasmic projections significantly influence the hemodynamic resistance to blood flow. To examine mechanotransduction mechanisms, we investigated *in vivo* the hemodynamic conditions in capillaries that control endothelial pseudopod formation.

Materials and Methods—Capillaries in rat skeletal muscle were fixed under carefully controlled perfusion conditions. The formation of endothelial pseudopodia were observed in cross-sections with electron microscopy and quantified with differential interference contrast microscopy under physiological, stasis, and reperfusion flow conditions.

Results—Application of physiological levels of fluid flow prevents capillary endothelium to project pseudopodia into the capillary lumen. Reduction of fluid flow to near zero promotes the incidence of pseudopod projection from 5% to 55% of capillaries. After capillary pseudopodia have formed under static conditions, about one-half retract upon restoration of fluid flow. The presence of red blood cells in the capillary lumen prevents pseudopod formation.

Conclusions—The results suggest that there is a mechanism that serves to control cytoplasmic projections in capillary endothelium that is under the control of hemodynamic fluid stress. Investigation of pseudopodia growth on endothelial cells may be significant in understanding capillary obstruction in cardiovascular diseases.

Keywords

skeletal muscle; gracilis muscle; microcirculation; actin polymerization; rat; endothelium; pseudopod formation; cytoskeleton

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In capillaries, the shapes of endothelial cells have a direct impact on blood flow. As the narrowest vessels, even slight changes in endothelial cell shape, swelling, or a shift in their membrane adhesive properties may have a direct impact on capillary perfusion [24]. In spite of the importance of endothelial shapes for microcirculation, few studies have addressed the issue in more detail [14].

In addition to passive viscoelastic deformation [30,31], endothelial cells have the ability to spontaneously change their shape, for example, during phagocytosis, cell migration, or angiogenesis [1,2,4,15,19,20,34–36]. In particular, they have the ability to project cytoplasmic projections into the lumen of capillaries [10]. Even though these projections are relatively small (of the order of 1 μm in length), the narrow lumen dimensions of capillaries ($\sim 5 \mu\text{m}$) set up a situation in which even relatively short projections can have a major impact on capillary velocity and flow rates. In the presence of endothelial cytoplasmic projections into the capillary lumen, both the flow of plasma is impaired, and erythrocytes and leukocytes may produce an increase in resistance to flow [10], possibly leading to virtual occlusion of the capillary lumen [23]. Little is known about what causes the formation of cytoplasmic projections and what mechanism serves to minimize their formation in normally perfused capillaries.

In this study, we examine the role of hemodynamic stresses on the formation of cytoplasmic projections by endothelial cells. The current study was designed to investigate the effect of fluid shear stress under *in vivo* conditions. We provide evidence that physiological fluid shear stress in capillaries of rat skeletal muscle serves to prevent the formation of cytoplasmic projections. Conditions of near zero fluid shear stress in the absence of plasma proteins may be associated with the formation of endothelial pseudopods.

MATERIALS AND METHODS

Animals

The animal procedures were approved by the University of California—San Diego Animal Subjects Committee. Male Wistar rats (350–400 g; Charles River Laboratories, Wilmington, Massachusetts, USA; $n = 18$) were housed in a controlled environment and maintained on a standard pellet diet for at least three days before the experimental procedures. The animals were administered general anesthesia (sodium pentobarbital, 50 mg/kg intraperitoneally [i.p.]; Abbott Laboratories, North Chicago, IL, USA) supplemented upon reflex testing to a leg pinch (5 mg/kg intravenously [i.v.]).

Microvascular Perfusion

The mid-segment of the *gracilis anticus* muscle was hemodynamically isolated *in situ*, as previously described [33], and perfused via catheters in the femoral artery and vein (PE 50; Clay Adams, Parsippany, NJ, USA). The muscle segment is located between the descending artery, a branch of the femoral artery in the groin area at the proximal end of the muscle, and the femoral artery and saphenous vein at the distal region of the muscle close to the knee (Figure 1).

Upon cannulation, the arterial inflow and venous outflow were immediately connected to a reservoir with heparinized isotonic saline to maintain continuous perfusion during microsurgery. A number of smaller vessels to the muscle segment were surgically ligated (4-0 silk; Ethicon, Sommerville, NJ, USA). Since capillaries inside the muscle are too small and numerous for ligation, the distal and proximal ends of the muscle segment were occluded by placement of clamps (Figure 1). This procedure prevented any capillary blood flow between the isolated muscle segment and the surrounding muscle.

The inflow pressure of the perfusates, via the femoral artery catheter, was adjusted between 0 and 120 mmHg by hydrostatic pressure and monitored throughout the duration of the experiments. The venous outflow pressure was kept at 0 mmHg (relative to the atmospheric pressure).

Hemodynamic Isolation and The Hidden Arteriole

After cannulation of the femoral artery, the controlled input pressure was maintained at 120 mmHg for 25 minutes in all muscle specimens. The aim was to clear the muscle microcirculation of blood cells and generate a near cell-free venous flow. The clear venous output served as an indication of an isolated muscle microcirculation that is only perfused from the controlled input of the catheter at the femoral artery.

In a pilot set of experiments (shown in group II), the venous outflow appeared slightly bloody by visual inspection (Table 1) and contained red blood cells (RBCs) up to an estimated hematocrit of 5% (compared to a known hematocrit solution) (data not shown), even though only heparinized saline was perfused through the femoral input; a clear output could only be achieved by cardiac arrest via euthanasia (Nembutal®, MWI Veterinary Supplies, Meridian, ID; 120 mg/kg). Further examination showed that RBCs in the venous outflow were due to a hidden arteriolar connection positioned along the descending artery, as described in the literature [32]. The hidden arteriole, a small vessel of ~200 μ m in diameter in an adult rat, permits collateral blood flow from the arteriolar arcade network of the underlying skeletal muscle into the descending artery of the gracilis muscle (Figure 1). The hidden arteriole bifurcates at a right angle to the muscle surface and is not visible without the separation of the descending artery from the muscle tissue [32]. The separation was avoided in the pilot set of experiments to minimize trauma to the muscle, but was carried out in the following experiments to permit ligation of the hidden feeder artery.

After ligation of the hidden arteriole by separating the muscle segment from the underlying connecting tissue, the only input with controlled pressure in the physiological range to the muscle microcirculation was via the femoral artery catheter. Our experiments that included this procedure confirmed the isolation of the *gracilis* muscle segment via a clear fluid output (as determined by unaided eye inspection) from the femoral vein catheter within 10 minutes of saline perfusion. Comparison with a known hematocrit solution, showed that the clear venous output fluid contained less than 1% hematocrit. Perfusion without a collateral input is a requirement to generate a near zero flow in capillaries, when arterial pressure and venous pressure are adjusted to the same value [32].

Perfusates and Perfusion Pressure

Two sets of perfusion criteria were selected to examine the influence on capillary pseudopod formation: 1) the contents of the perfusion medium at physiological blood pressure and 2) the effect of arterial blood pressure and, therefore, the shear stress on the capillary wall. Set (a) contained groups I, II, III, and IV, which varied in perfusate contents, and set (b) contained groups III, V, and VI, which varied in perfusion pressure (Table 1). At the start of all experiments, a control flow period of 25 minutes with heparinized isotonic saline was applied.

As a control for perfusate contents in the experimental set (a), autologous perfusion (autologous blood; group I) of the isolated muscle segment was used. Experiments with collateral flow from the hidden arteriole were placed into a separate group II (saline with collateral flow). Three different inflammatory stimuli were added to the cell-free heparinized isotonic saline in group IV (cell-free saline with inflammatory stimuli): 2.5 mg endotoxin (*Escherichia coli*-derived endotoxin, LPS, serotype 127:B8; Sigma Chemical Company, St. Louis, MO, USA),

hydrochlorpromazine (20 μ M, C₁₇H₂₀Cl₂N₂S; Sigma), and platelet-activating factor (PAF, 10⁻⁹ M; Sigma).

As a control for perfusion pressure in the experimental set (b), heparinized isotonic saline (cell-free saline; group III) was used in the isolated muscle segment under physiological blood pressure (120 mmHg input pressure). In group V (stasis with cell-free saline), the control flow period was followed by a complete cessation of arterial inflow and stasis for 45 minutes before tissue fixation. Stasis was defined as no visible flow output or input under physiological pressure from microvessels or the catheter. Group VI (reperfusion with saline after stasis) received the same treatment as in group V. However, following 45 minutes of stasis, the tissue was reperfused at the same flow rates as before stasis. Reperfusion was applied for 30 minutes at physiological input pressure before tissue fixation.

Tissue Fixation and Preparation

Capillary endothelial shapes were arrested by superfusion of the fixative before excising the muscle segment. During superfusion fixation, a controlled arterial input flow to the muscle segment was maintained in all experiments except the stasis group. Only in the stasis group (V), the input to the *gracilis* muscle remained occluded with zero flow during tissue fixation.

The muscle tissue was initially fixed with a 4% formaldehyde solution in 0.1 M of sodium cacodylate buffer (310 mOsm, pH 7.4). The fixative was topically applied on the muscle tissue *in situ* for two hours (superfusion fixation), which provides adequate penetration of the fixative throughout the thickness of the muscle [10]. The superfusion fixation with formaldehyde serves to arrest the shape of the endothelial cells and their pseudopodia in capillaries of the superficial muscle layers within seconds [29]. Following the primary drip fixation, the entire muscle segment shown between the clamps in Figure 1 was excised and placed into 1.5% glutaraldehyde (Polysciences Inc., Warrington, PA) in 0.1 M of sodium cacodylate buffer (310 mOsm, pH 7.4) for one hour. After the fixation process, the tissue was cut into 8–10 small rectangles (~2 × 1 × 1 mm depth), with the longer side parallel to the muscle fibers. Only the superficial layers of the muscle tissue, to a depth of about 1 mm, were processed to avoid the possibility of incomplete tissue fixation *in situ*. The tissue blocks were post-fixed in 1% osmium tetroxide (Polysciences Inc., 310 mOsm, pH 7.4) for two hours and slowly dehydrated in multiple steps of increasing alcohol concentrations. Due to the use of isotonic fixatives and a slow dehydration protocol, tissue shrinkage was minimized and vessel proportions were maintained within 96% during sectioning and staining, as shown by Lee et al. [10]. The tissue was counterstained with 2% uranyl acetate and 1.3% Reynolds' lead citrate and embedded in low-viscosity resin (Spurr epoxy embedding medium; Ted Pella, Inc., Redding, CA, USA). Tissue sections were cut from each block without the preparation of serial sections on any block; this sectioning strategy results in single random sections throughout the muscle. One *gracilis* muscle segment was excised per animal.

Microscopy

The resin blocks were sectioned (LKB microtome; Stockholm, Sweden) perpendicular to the muscle fibers into semithin sections (1 μ m) for light microscopy and into ultrathin sections (70 nm) for electron microscopy (EM). Semithin sections were stained with toluidine blue and examined with an Axiovert 40 inverted light microscope (Carl Zeiss, Frankfurt, Germany) using differential interference contrast (DIC) at 1000X optical magnification (100 × oil objective, 1.4 numerical aperture). DIC microscopy enhanced the contrast along cell edges, which made it possible to detect pseudopodia protrusions from endothelial cell surfaces (with a width of 0.1–0.2 μ m) near light microscopy resolution limits. The optical resolution possible with DIC technique is 0.1 μ m. DIC microscopy also allowed a reliable recording of large numbers of pseudopodia in each section, which would be impossible with EM. Digital DIC

images were recorded by a charge-coupled device (CCD) camera. One hundred twenty semithin sections from 18 different animals were analyzed with DIC microscopy with about 100 capillary cross-sections per section. Measurements were expressed as the percentage of capillaries sampled that contain either plasma, RBCs, or pseudopodia.

Selected ultrathin sections were stained with lead citrate and osmium tetroxide and examined with an electron microscope (EM10; Carl Zeiss) at 50,000X magnification. Typical endothelial pseudopod features, such as the elongated shape and the presence of plasmalemmal membrane [10], confirmed pseudopod formation at the EM magnification.

Endothelial Pseudopodia Analysis

To be classified as a pseudopod, the cytoplasmic extension into the capillary lumen had to exceed the average thickness of the endothelial cell forming the capillary wall (Figure 2). Such cytoplasmic extensions may be positioned at the intercellular junction (arrows in Figure 3), or they may be present at sites on the capillary endothelium between junctions. The base of the pseudopod was considered the inner surface of the capillary wall.

Two independent operators, blinded with respect to the experimental groups, analyzed the capillary cross-sections for the detection of pseudopodia.

Statistics

Measurements are presented as the mean $\pm$ standard error of the mean. Statistical comparisons for pseudopod formation between the experimental groups were analyzed by using the Kuskal-Wallis analysis of variance (ANOVA) test. A value of $p < 0.05$ was considered significant.

RESULTS

The ultrastructural studies (EM imaging) showed that many pseudopodia originate at endothelial junctions (Figure 3, arrows) and protrude from the inner endothelial wall into the capillary lumen. In the majority of cases, the pseudopodia pointed toward the center of the capillary lumen. In some cases, however, the direction was almost parallel to the endothelial wall. The pseudopodia contained a homogeneous actin matrix and were depleted of all cytoplasmic organelles, membrane caveolae, or other membrane pits (Figures 2 and 3). On average, the minimum length of a pseudopodium was greater than 1 μm , which was clearly distinguishable from capillaries without pseudopod formation (Figure 3A). Most pseudopodia measured 1–2 μm in length (Figure 3B), whereas longer versions extended up to 5 μm into the lumen of the capillary, reaching across almost the entire lumen (Figure 3C). The width of pseudopodia was typically between 0.1 and 0.2 μm (Figure 2).

Capillary cross-sections from DIC images were separated into three categories, depending on the appearance of the capillary lumen. Examples of each category are shown in Figure 4: capillaries showing either plasma without evidence of pseudopodia formation (Figure 4A), RBCs present (Figure 4B), or pseudopodia protruding into their lumen (Figure 4C).

Quantitative analysis of all cross-sections in DIC images (Figures 5 and 6) showed that capillaries perfused under physiological pressure (groups I–IV) only revealed protrusion characteristics of endothelial pseudopodia in 5% or less of the cross-sections. All groups showed a fraction of capillaries containing RBCs in their lumen. No endothelial pseudopod formation was observed when RBCs were present in the capillary lumen. A comparison between the effects of the perfusion pressure (Figure 5) and the contents of the perfusion medium (Figure 6) showed that the arterial perfusion pressure had a strong influence on the formation of pseudopodia in capillaries, while the perfusion medium itself had only a minor effect.

Perfusion of the *gracilis* muscle at physiological blood pressure (Figure 5, group III, control) showed 5% capillary cross-sections with pseudopodia formation. The percentage of capillaries with pseudopodia increased to 55% when near-zero-flow conditions were established in the capillary network (group V, stasis). Upon restoration of capillary flow for 30 minutes (group VI, reperfusion), the percentage of capillary cross-sections with pseudopodia was reduced to 28% (Figure 5). Heparinized isotonic saline was used as the perfusion medium in all experiments with pressure change. Changes in perfusion pressure (Figure 5) from the experimental set (b) had a statistical significant effect on pseudopodia formation, using the ANOVA test (comparisons between group III, V, and VI showed $p < 0.01$).

Changes in perfusion medium, while the inflow pressure was maintained at physiological levels, caused no increase of pseudopodia formation above 5% (Figure 6). Even the addition of endotoxins to the perfusing media as inflammatory stimulus for endothelial cells (group IV, cell-free saline with inflammatory stimuli) did not increase the percentage of capillary cross-sections with pseudopodia (Figure 6). Statistical comparison between groups I–IV in the experimental set (a) did not show any significant differences in the frequency of capillaries with pseudopodia ($p > 0.05$).

A large fraction of RBCs was observed in all muscle segments, including cases in which the femoral venous output was clear, suggesting a near cell-free vessel network. This result led to further examination of the images to detect possible spatial distribution patterns of RBCs in local areas of nonperfused muscle segments during the fixation process, which would possibly affect pseudopod formation in these areas. DIC images of muscle cross-sectional domains (170 μm in diameter) with an average of 30 capillaries per domain were counted. Figure 7 shows scatter diagrams of the fraction of capillary cross-sections with pseudopodia and with RBCs in the same domain. Each point in the scatter graph represents the value in one domain. The controlled perfusion (group III), near stasis (group V), and reperfusion (group VI) experiments were analyzed. All measurements fell below the broken identity line, reinforcing the observation that pseudopodia formation was not found in any capillary that contained a RBC. The scattering of domains along the length of the x-axis demonstrates that RBCs were present in all domains of the muscle segment. During stasis (group V), a significant increase of the number of pseudopodia was observed, shifting the domains (points in the scatter graph) upward when compared to the control group (III). Upon reperfusion (group VI), approximately half of the muscle domains shifted back toward the distribution pattern observed in the control group (III) with a lower percentage of pseudopodia. The remaining muscle domains in the reperfusion group (VI) resided in the upper area of the scatter graph, maintaining an elevated level of pseudopod formation, as observed under stasis conditions (group V).

DISCUSSION

The current results show that even though capillary endothelial cells in rat skeletal muscle can undergo active deformation and project pseudopods, fluid flow serves to prevent their formation. The capillary lumens are almost completely circular, optimizing the normal passage of blood cells even in the presence of acute inflammatory mediators. Reducing the flow to the level of stasis, leads to the formation of endothelial pseudopodia, some of which protrude far into the capillary lumen. Once pseudopodia have formed, their length and number can be reduced by the reintroduction of fluid flow.

Cytoplasmic projections are the basis for active deformation of the endothelial cells as required for migration, phagocytosis, and possibly angiogenesis [2,4,5,12,15,25,35,36]. Cytoplasmic projections in capillaries tend to be sheet-like, as compared to finger- or spike-like projections, and tend to frequently originate at endothelial junctions [8,10]. Depending on the length of the

projection, they have a profound effect on capillary hemodynamic resistance by disrupting the velocity profile of the normal circular capillary lumen [1,10].

The fact that the reperfusion of capillaries serves to retract at least some of the pseudopodia implies the presence of a reversible dynamic mechanism in the cytoskeleton of endothelial cells, which has yet to be identified. A similar retraction mechanism was also observed in experiments investigating circulating leukocytes, the results of which demonstrated that low levels of fluid shear stress (about 1 dyn/cm²) stimulated pseudopod retraction [16].

There is extensive evidence that endothelial-cell shapes are under the control of fluid shear stress not only in terms of cell elongation, but also in terms of direction of migration [3,6,7, 11,17,18,28]. Most of this evidence derives from observations of endothelial cells in large vessels or in culture in a flow chamber. This suggests that fluid shear stress may be a major stress component controlling active cell deformation, even though shear-stress values may be small (of the order of 1–10 dyn/cm²) compared to the normal stress component of fluid flow (e.g., pressure at 10 mmHg = 13,330 dyn/cm²). It has been shown in the literature that normal fluid stress may affect endothelial structure [21,26,27,36], but there is currently no evidence that even high values of normal fluid stress alone affect pseudopod formation directly. In contrast, small values of fluid shear stress (<5 dyn/cm²), more prominent in small vessels such as capillaries, seem to play a more important role on pseudopodia formation. Our study indicates that low levels of fluid shear stress, as applied in collateral flow experiments (group II) with minimal flow input, serve to prevent an increase in pseudopod formation. In the presence of even such low levels of fluid-shear stress, the incidents of pseudopodia formation remained the same as under physiological flow conditions (Figure 6) and did not increase as in stasis conditions (Figure 5).

Our results indicate that pseudopodia develop in near-zero shear-stress conditions. Under normal physiological conditions, true zero flow can only be generated in a skeletal muscle without collateral flow. However, collateral flow is present in all healthy muscle tissues to bypass any possible occlusion of a major feeding vessel. The conditions under which pseudopodia formation appear in low-flow circumstances, which this study was investigating, may be critical in conjunction with other conditions associated with inflammation or cardiovascular diseases that promote pseudopodia formation in capillaries. In such an affected area, it is, therefore, possible that static flow conditions can complicate the situation by pseudopod formation in capillaries that may impact the passage of blood cells even more and may contribute to local occlusion of the capillary blood flow. This effect enhances ischemic regions of the microcirculation and may explain progressive conditions seen in stroke, shock, or heart attack [23,38,39], if flow cannot be reestablished within an hour or less. However, as demonstrated in this study, single inflammatory stimulators, such as endotoxins, may not significantly affect the formation of pseudopodia in capillaries of otherwise healthy skeletal muscle.

During angiogenesis, the formation of intraluminal thin septa in highly activated, newly formed capillaries have been reported under long-term treatment with prazosin, a vasodilator, which promotes elevated blood flow [4]. These thin septa may form as a result of pseudopodia growth across the lumen of newly forming vessels, which are significantly smaller in diameter than untreated mature capillaries [37]. In contrast to our current observations, Zhou et al. reported that the pseudopodia seem to originate mostly from the plasma membrane of the cell bodies of ECs at a distance from cellular junctions [37] and would have to grow across the lumen of a capillary in spite of the fluid shear stress imposed by blood flow. It is possible that pseudopodia growth in capillaries may occur under high- or low-flow conditions and could indicate a cytoplasmic reaction of ECs to flow levels other than the normal physiological level. Our current study did not look at levels of elevated blood flow and shear stress, but found—

at near-zero-flow conditions—pseudopod formation rapidly increasing to a level of 55% of all counted mature capillaries, frequently located at cell junctions, as seen in tissue-culture experiments [8] or under conditions associated with hypoxia and ischemia [38,39]. It seems possible that the underlying cause may be a cytoplasmic reaction of the ECs designed to reduce the capillary lumen via pseudopodia growth. This might be an attempt to increase the flow in near-stasis conditions and to reestablish physiological levels of hemodynamic stresses, resulting in equilibrium of flow and vascular diameter, as described by Masuda et al. [13]. In any case, the observation of pseudopodia growth *in vivo* under near-zero flow may influence the outcome of a pathological condition and may affect a possible recovery. More studies need to be performed to determine by what mechanisms ECs form pseudopodia under these conditions.

Analysis of local domains of rat skeletal muscle indicates a random spatial distribution of RBCs in capillary cross-sections in all muscle segments independent of pseudopodia formation (Figure 7). We could not detect local RBC shunting in muscle areas down to a domain size of ~30 capillary cross-sections (170 μm in diameter). Zero-flow input pressure did not seem to promote RBC shunting in localized tissue areas. Pseudopodia formation in capillaries was not due to a shunting effect in nonperfused domains in the muscle tissue.

The scatter plot (Figure 7) generated by the local domain analysis of the reperfusion group (group VI) has a similar appearance to a superposition of the graphs from the controlled perfusion group (group III) and the stasis group (group V). Since the reperfusion group was a continuation of the same procedures performed in the previous experiments (control group III and stasis group V), this pattern indicates a shift of approximately half of the domains back from high pseudopodia incidence to the original, lower incidence of pseudopodia in the control group. This shift supports the observation that pseudopodia formation in capillaries generated under stasis conditions can be reversed by reperfusion. The mechanism causing some pseudopodia to remain in the capillary lumen requires further investigation.

The mutual exclusion between the presence of RBCs and pseudopod projection in the same capillary lumen was a consistent finding in this study, suggesting that RBCs may have a powerful mechanism to prevent endothelial pseudopod formation. However, the mechanism remains unknown. A recent report dealing with leukocytes shows that pseudopod formation can be prevented under fluid-shear conditions only in the presence of RBCs, but not in their absence [9]. The effect of the RBCs on pseudopod formation is mostly due to scavenging of oxygen free radicals and less due to the rheological properties of RBCs [9].

In this study, we used cell-free saline as the perfusion medium to investigate the effects of different perfusion pressures while eliminating the direct influences of RBCs on pseudopod formation and capillary obstruction. In the absence of the majority of RBCs, the luminal membrane of endothelial cells forming the wall of the capillary is directly exposed to the applied fluid-shear or stasis condition without the ability to scavenge oxygen free radicals or NO, which are possible mediators released by RBCs. Thus, it is possible that capillary endothelial cells during stasis may exhibit a shift in their membrane mechanotransduction mechanism that controls pseudopod formation [8,10,25].

RBC shunting as an explanation for the presence of RBCs in a fraction of capillaries in all of the experimental groups, and as a possible cause for pseudopodia formation in capillaries, is not supported by our results. The appearance of RBCs in all experimental groups, even though the venous output appeared clear, indicates that some RBCs might have been trickled into the microcirculation of the muscle segment, despite the occlusion of all visible input vessels. Less than 1% hematocrit, indicated by a clear venous output, could still be circulating in the isolated muscle segment during perfusion with heparinized saline. The input pressure of the saline

perfusion medium may move RBCs through the microcirculation and evenly distribute them over the entire muscle segment upon fixation, as seen in the domain analysis (Figure 7) without an indication of shunting. Only in the stasis group (Figure 5) was the RBC content lowered to 18% RBCs because the input pressure was close to zero. Return of the input pressure to physiological levels in the reperfusion group can explain the appearance of about the same levels of RBC inflow as seen before the stasis condition (Figure 5). Though a small amount of RBCs may be flowing in or out of the isolated muscle segment at a single time point (less than 1% hematocrit), over the period of many minutes under physiological pressure, the RBCs accumulate in the capillary network of the isolated muscle segment. This could explain a relatively high frequency of capillaries with RBCs visible after fixation in both experimental set-ups that have a physiological input pressure from the catheter (groups III and VI in Figure 5). Only in the stasis group (group V in Figure 5) did a near-zero flow pressure reduce the inflow and distribution of RBCs across the capillary network of the isolated muscle segment. A 1% hematocrit contributes relatively little to the fluid-shear stress at the physiological flow input. Therefore, we could study the effects of pure fluid-shear stress exposure in most of the controlled perfused capillaries and in individual endothelial cells.

The formation of pseudopodia appears to be due to an actin assembly process in individual endothelial cells [8,22]. Biochemical analysis of isolated pseudopodia from mouse NIH-3T3 cells suggest that cytoskeletal-associated proteins are present in different patterns in purified pseudopodia as compared to the main cell body [22]. There is a significant difference in the signaling pathway in growing and retracting pseudopodia, indicating that there may be, besides shear stress, a possible biochemical control of pseudopodia growth *in vivo* [22].

The results of this study, however, show that the incidence of pseudopod formation dramatically increases from 5% in physiological perfused tissue to 55% in near-stasis flow conditions, if untreated. The reintroduction of normal fluid flow served to reduce the presence of obstructive pseudopodia in approximately one-half of the capillaries. Nevertheless, 28% of the capillaries were still found to have pseudopodia in their lumen after the reperfusion for 30 minutes. It is possible that pseudopodia may be completely retracted if reexposed to physiological flow for longer periods of time than were achieved in this study.

The current study was designed to investigate the effect of fluid shear stress under *in vivo* conditions. It remains to be determined to what degree other factors, such as blood and plasma viscosity, or hematocrit, influence the phenomenon. It needs to be investigated whether fluid-shear stress or shear-rate control the pseudopod formation. Nevertheless, the phenomenon that the incidents of pseudopodia formation can be reduced with the application of physiological fluid flow after stasis have not been described before.

CONCLUSIONS

In conclusion, the current experiments show that capillaries produce pseudopodia under acute conditions. The incidence of pseudopod formation increases during acute periods of stasis with near-zero flow. The reintroduction of normal flow after stasis reduces the presence of obstructive pseudopodia. Even low flow rates (e.g., in the presence of collateral flow), as well as the presence of RBCs, reduce the incidence of pseudopod formation in capillaries. These results suggest that pseudopod formation is a reversible mechanism, dependent on exposure to fluid flow. In shock or stroke situations, this microeffect may have a major impact on the recovery process of tissue perfusion. The investigation of the cause and prevention of pseudopodia growth may be significant in understanding capillary obstruction in cardiovascular disease.

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Figure 1. Schematic diagram of the surgical procedure to perfuse a separated segment of the *gracilis anticus* muscle in rats. The hidden arteriole (white circle underneath the femoral artery) forms a collateral connection with the arteriolar arcade network of the underlying skeletal muscles. The diagram is modified from Lee et al. (10).

Figure 2.

Electron micrograph of an endothelial pseudopod in a capillary lumen. By definition, to be considered as a pseudopod, its length must exceed the average thickness of the endothelium forming the capillary wall by a minimum of 1 μm . A typical pseudopod width is about 0.1–0.2 μm .

Figure 3.

Electron micrographs of capillary cross-sections in gracilis anticus muscles of rats. (A) Without pseudopodia formation, (B) with frequently observed shorter pseudopodia (1–2 μm), and (C) with longer pseudopodia up to 5 μm into the capillary lumen. Many pseudopodia appear at cellular junctions (arrows).

Figure 4. Examples of differential interference contrast images of capillary cross-sections in the gracilis anticus muscle of rats. (A) Capillary lumen showing plasma without evidence of pseudopodia formation, (B) in the presence of a red blood cell, and (C) with endothelial pseudopodia protruding into the capillary lumen.

Figure 5. Quantitative analysis of capillary cross-sections from differential interference contrast images. Effects of perfusion pressure on pseudopodia formation in capillaries. Under stasis conditions (group V), a significant increase of pseudopodia formation can be observed. Upon reperfusion (group VI), about one-half of the pseudopodia formation is reversed.

Figure 6.

Quantitative analysis of capillary cross-sections from differential interference contrast images. Effects of contents of perfusion media on pseudopodia formation in capillaries. No increase of pseudopodia formation above 5% can be observed.

Figure 7.

The effects of spatial distribution of red blood cells (RBCs) on pseudopodia formation in capillaries. Scatter-plot graphs with average trend lines (unbroken identity lines) of the frequency of capillary cross-sections with pseudopodia (ordinate) and with red blood cells (RBCs) (abscissa*) in domains of skeletal muscle tissue. The analysis was carried out on differential interference contrast images, and each point represents a value derived from a single muscle tissue domain of $170\ \mu\text{m}$ in diameter, containing about 30 capillary cross-sections. All measurements fall below the broken identity line, reinforcing the observation that pseudopodia formation was not found in any capillary that contained an RBC. The graph from the reperfusion group (group VI) has a similar appearance to a superposition of the graphs from the controlled perfusion group (group III) and the stasis group (group V).

*Scale is plotted reversed (from 100 to 0).

Table 1

Experimental animal groups

Group	Number of animals (n)	Collateral flow	Perfusion time	Arterial input	Output appearance	Experimental criteria
I: Autologous blood control	2	Yes	25 minutes	120 mmHg	Bloody	(a) Perfusate contents
II: Saline with collateral flow	4	Yes	25 minutes	120 mmHg	Slightly bloody	
III: Cell-free saline control ^a	3	No	25 minutes	120 mmHg	Clear	
IV: Cell-free saline with inflammatory stimuli	3	No	25 minutes	120 mmHg	Clear	
V: Stasis with cell-free saline	3	No	25 minutes 45-minute stasis	120 mmHg 0	Clear —	(b) Perfusion pressure
VI: Reperfusion with saline after stasis	3	No	25 minutes 45-minute stasis 30 minutes	120 mmHg 0 120 mmHg	Clear — Clear	

^a Group III was used as the control for (b).