Shear-Sensitive Regulation of Neutrophil Flow Behavior and Its Potential Impact on Microvascular Blood Flow Dysregulation in Hypercholesterolemia

Xiaoyan Zhang,* Ran Cheng,* Dylan Rowe, Palaniappan Sethu, Alan Daugherty, Guoqiang Yu, Hainsworth Y. Shin

Objective—Shear stress–induced pseudopod retraction is an anti-inflammatory measure that minimizes neutrophil activity and is regulated by membrane cholesterol. We tested the hypothesis that a hypercholesterolemic impairment of shear mechanotransduction alters the neutrophil flow behavior leading to microvascular dysfunction.

Approach and Results—We examined the shear effects on the flow behavior of human leukocytes. When subjected to shearing during cone-plate viscometry, leukocyte suspensions exhibited parallel time-dependent reductions in viscosity and pseudopod activity. Shear-induced reductions in suspension viscosity were attenuated by membrane cholesterol enrichment. We also showed that enhanced pseudopod activity of leukocyte suspensions in 10% hematocrit significantly ($P<0.05$) raised the flow resistance of microvascular mimics. These results implicate an impaired neutrophil pseudopod retraction response to shear in hypercholesterolemic microvascular dysfunction. We confirmed this using near-infrared diffuse correlation spectroscopy to assess skeletal muscle blood flow regulation in the hindlimbs of mice subjected to reactive hyperemia. Using a custom protocol for the mouse, we extrapolated an adjusted peak flow and time to adjusted peak flow to quantify the early phase of the blood flow recovery response during reactive hyperemia when shear mechanobiology likely has a maximal impact. Compared with mice on normal diet, hypercholesterolemic mice exhibited significantly ($P<0.05$) reduced adjusted peak flow and prolonged time to adjusted peak flow which correlated ($r=0.4$ and $r=0.3$, respectively) with neutrophil shear responsiveness and were abrogated by neutropenia.

Conclusions—These results provide the first evidence that the neutrophils contribute to tissue blood flow autoregulation. Moreover, a deficit in the neutrophil responsiveness to shear may be a feature of hypercholesterolemia-related microvascular dysfunction. (Arterioscler Thromb Vasc Biol. 2014;34:587-593.)

Key Words: inflammation ● mechanotransduction, cellular ● microcirculation ● optical devices ● regional blood flow

Although hypercholesterolemia is a major risk factor for heart disease and stroke, the link between plasma cholesterol levels and incidence of cardiovascular pathology is not without controversy. In addition to its pathological effects on large vessels, hypercholesterolemia promotes microvascular dysfunction. Efforts to link microvascular dysfunction to hypercholesterolemia focused on its detrimental impact on the endothelial regulation of arteriolar vasoactivity. However, endothelium-mediated vasomotor control is not the sole determinant of tissue perfusion. Other factors that impact hemodynamic resistance are plasma lipid concentrations and blood cell rheology, both of which affect blood viscosity.

Neutrophils, although having little effect on large vessel blood flow, influence blood rheology in the microcirculation where vessel diameters (4–100 μm) are similar to their cell dimensions. Once activated, neutrophils project pseudopods and bind to other cells, such as other leukocytes, endothelium, and platelets, all of which hinder their passage through the microvasculature and raise peripheral resistance. Thus, neutrophil activation profoundly affects microvascular flow.

Under physiological conditions, neutrophil activity is restricted by various anti-inflammatory factors. Among these, there is compelling evidence from human and rodent studies that shear stress is anti-inflammatory for neutrophils. Specifically, acute exposure of neutrophils to shear reduces their pseudopod activity, F-actin content, and surface expression of CD18 integrins. By doing so, shear stress mechanotransduction serves as a control mechanism that ensures neutrophils adopt a rounded, deformable, and nonadhesive state so as to minimize their impact on peripheral resistance.
**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APF</td>
<td>adjusted peak flow</td>
</tr>
<tr>
<td>CH</td>
<td>cholesterol:methyl-β-cyclodextrin complexes</td>
</tr>
<tr>
<td>DCS</td>
<td>diffuse correlation spectroscopy</td>
</tr>
<tr>
<td>HFD</td>
<td>high-fat diet</td>
</tr>
<tr>
<td>ND</td>
<td>normal diet</td>
</tr>
<tr>
<td>rBF</td>
<td>relative changes of blood flow</td>
</tr>
<tr>
<td>RH</td>
<td>reactive hyperemia</td>
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<tr>
<td>T&lt;sub&gt;adj&lt;/sub&gt;</td>
<td>time to adjusted peak flow</td>
</tr>
</tbody>
</table>

This possibility is in line with reports<sup>13,14</sup> that an impaired neutrophil shear response raises microvascular resistance.

Notably, an impaired control of neutrophil pseudopod activity by shear stress develops early during development of a hypercholesterolemic blood state in mice fed a high-fat diet (HFD).<sup>15</sup> Considering the link between neutrophil pseudopod activity and tissue blood flow, we hypothesized that a deficit in membrane cholesterol-related regulation of neutrophils by shear contributes to microvascular dysfunction in hypercholesterolemia.

We used real-time viscometry of leukocyte suspensions and microfluidics to link microvessel resistance to shear regulation of neutrophil flow behavior. We also used low-density lipoprotein receptor–deficient mice fed a normal diet (ND) or HFD to reveal a first correlative link between neutrophil shear sensitivity and in vivo tissue blood flow regulation. For this purpose, we tested the perfusion recovery responses of the posterior thigh muscles of mice subjected to transient blood flow occlusion (ie, reactive hyperemia [RH]). Conceivably, neutrophils in tissues undergoing RH experience a no-flow situation that mildly activates them<sup>16</sup> because of upstream blood flow occlusion followed by an acute exposure to shear during reperfusion. This scenario implicates neutrophil shear sensitivity as a component of RH.

To relate changes in tissue blood flow autoregulation to altered neutrophil shear sensitivity, we used a novel optical technology: near-infrared diffuse correlation spectroscopy (DCS).<sup>17</sup> This technique provided noninvasive, real-time tissue blood flow measurements deep in the murine thigh muscle to detect the neutrophil impact on the in vivo dynamics of RH. The combined use of classical cell biomechanics methods, current microfluidics approaches, and state-of-the-art optical spectroscopy revealed novel mechanistic insight regarding hypercholesterolemic microvascular dysfunction and the neutrophil.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**Shear-Induced Pseudopod Retraction Impacted Flow Behavior of Neutrophils in Suspension**

To assess effects of shear stress on leukocyte flow (ie, tumbling) behavior, the suspension viscosities of leukocyte-enriched plasma, mildly stimulated with 10 nmol/L f-Met-Leu-Phe, were examined during 10-minute exposure to cone-plate shear flow. At the initial time point (t=30 seconds after flow onset), leukocyte-enriched plasma exhibited significantly (P<0.05) higher viscosities compared with cell-free plasma (Figure 1A). Although viscosities of cell-free plasma remained constant, viscosities of leukocyte-enriched plasma decreased in a time-dependent manner during viscometry (Figure 1A). After 9 minutes, viscosities of leukocyte-enriched plasma were reduced to levels similar to those of cell-free plasma.

In separate experiments, the percentage of neutrophils in leukocyte-enriched plasma that displayed pseudopods (Figure 1 in the online-only Data Supplement) decreased in a time-dependent fashion under shear with significant (P<0.05) reductions detected after 1 minute of flow (Figure 1B). In contrast, the percentage of neutrophils with bound platelets in these cell populations increased under shear and plateaued after 5 minutes of flow at levels significantly (P<0.05) higher than those observed at the initial time point (Figure 1B). Moreover, cysteine protease inhibitor, E64, that blocks CD18 cleavage and enhances neutrophil-platelet binding under shear,<sup>18</sup> had no effect on the apparent viscosity of f-Met-Leu-Phe–stimulated leukocyte-enriched plasma throughout the duration of cone-plate viscometry. Viscosities of these cell suspensions were significantly (P<0.05) reduced after 2 minutes of shear in both the absence and presence of E64, relative to their initial viscosities at t=30 seconds (Figure 2).

**Cell Membrane Cholesterol Enrichment Altered Shear Stress Influence on Leukocyte Rheology**

Incubation of leukocytes with cholesterol:methyl-β-cyclodextrin complexes (CH) for all concentrations tested had no effect on their initial suspension viscosity at 30 seconds after flow onset (data not shown). Beyond this 30-second time point,
membrane cholesterol–enhancing agents dose dependently impaired shear-related reductions in leukocyte suspension viscosity (Figure 3). Specifically, leukocyte suspensions incubated with 0, 2, or 5 μg/mL CH exhibited significantly \((P<0.05)\) reduced viscosities after 4 minutes of cone-plate flow relative to their initial viscosities at \(t=30\) seconds. However, viscosity reductions for leukocyte-enriched plasma incubated with either 2 or 5 μg/mL CH were smaller relative to those for naïve cells. Moreover, viscosities of leukocyte-enriched plasma incubated with 10 μg/mL CH remained the same for the duration of viscosity measurements. The dose-dependent effect of membrane cholesterol enhancement was confirmed by linear regression analyses of the end point \((i.e., t=10\) minutes) viscosity measurements versus CH concentration. We detected a significant correlation \((R^2=0.94; P<0.05)\) between leukocyte suspension viscosity and CH concentration.

### Pseudopod Projection Influenced Resistance of a Microfluidics-Based Microvascular Mimic to Flow of Neutrophil Suspensions in the Presence of Red Blood Cells

We confirmed, using microfluidics, that the impact of neutrophil pseudopod projection on suspension viscosity influenced microvessel flow resistance. On injection of suspensions of purified neutrophils through a 50×500 μm microchannel at a constant flow of 1 mL/h, the pressure difference across the microfluidic channel was similar for perfusate containing either activated or inactivated cells (Figure 4A). Accordingly, there was no difference in microchannel flow resistance imposed by these 2 types of cell suspensions (Figure 4B). In the presence of 10\% hematocrit, perfusion of activated neutrophils enhanced pressure difference across the microchannel (Figure 4A) resulting in significant \((P<0.05)\) elevations \((17.5\%)\) in microchannel resistance relative to perfusion of nonactivated cells (Figure 4B). Separate experiments confirmed that the percentage of neutrophils with pseudopod(s) was significantly \((P<0.05)\) enhanced by stimulation with 10 nmol/L f-Met-Leu-Phe (Figure 4C and 4D).

**Neutrophils Contributed to Hypercholesterolemia-Induced Microvascular Dysregulation**

The blood flow recovery responses of hindlimbs of ND- and HFD-fed mice to 5-minute blood flow occlusion were assessed to explore the neutrophil contribution to hypercholesterolemic microvascular dysfunction. Compared with their ND-fed counterparts, low-density lipoprotein receptor–deficient mice fed a HFD exhibited significant \((P<0.05)\) time-dependent increases in plasma concentrations of free and total cholesterol (Table I in the online-only Data Supplement). For RH analyses, we ensured that during cuff occlusion, relative changes of blood flow \((rBF)\) values were <10\% relative to preocclusion levels for all animals tested (Table II in the online-only Data Supplement). We previously reported that successful blood flow restriction is repeatedly and reliably achieved once rBF, during cuff occlusion, reached <10\% of its preocclusion value.17 Notably, the rBF curves recorded for the posterior thigh muscles subjected to blood flow occlusion displayed a transient flow overshoot after cuff release followed by a return to baseline levels within 30 minutes (Figure 5A–5C). To quantify RH, all rBF measurements fluctuating within 10\% of the peak flow value were averaged and defined as adjusted peak flow \((APF)\). Using this approach, we did not observe differences in rBF (data not shown) or APF (Figure 5D) between mice on HFD or ND for 2 and 4 weeks. In contrast, rBF curves for mice fed a HFD for 8 weeks displayed a blunted peak overshoot compared with that of their ND-fed counterparts (Figure 5B). This blunting of rBF curves for 8-week HFD-fed mice manifested as significant \((P<0.05)\) reductions in APF relative to that for their ND-fed counterparts (Figure 5D). Markedly, acute depletion of ~90\% neutrophils from mice abrogated the effects of 8-week HFD on peak rBF and APF (Figure 5C and 5E).

**Figure 2.** Shear-induced reductions in cell suspension viscosity occurred independently of CD18-mediated neutrophil-platelet binding. Leukocyte-enriched plasma diluted 1:10 v/v in buffer was stimulated with 10 nmol/L f-Met-Leu-Phe in the absence (untreated [UT]) or presence of 28 μmol/L E64 and then subjected to cone-plate viscometry (shear rate: 450/s). Instantaneous viscosities of cell suspensions were examined and normalized to their initial viscosity at \(t=30\) seconds. Data are mean±SEM from \(n=3\) experiments. *, +, and #\(P<0.05\) compared with \(t=30\) seconds using 1-way repeated measures ANOVA with Dunnett’s method.

**Figure 3.** Membrane cholesterol enrichment altered shear-sensitive leukocyte rheological flow behavior. Leukocytes enriched with membrane cholesterol by incubation in 0 to 10 μg/mL cholesterol: methyl-β-cyclodextrin complexes (CH) were stimulated with 10 nmol/L f-Met-Leu-Phe and subjected to viscometry (shear rate: 450/s). A, Instantaneous viscosities of cell suspensions were monitored and normalized to the initial values at \(t=30\) seconds. B, The end point viscosities after 10-minute shear exposure were correlated with CH concentration. Data are mean±SEM from \(n=25\) experiments. *, +, and #\(P<0.05\) compared with \(t=30\) seconds using 1-way repeated measures ANOVA with Dunnett’s method.
To determine the temporal effects of neutrophils on RH, time points corresponding to the rBF measurements used to calculate APF were averaged and defined as time to APF (TAPF). Overall, median values of TAPF for ND-fed mice fell between 1 and 2 minutes after cuff release (Figure 5F). Notably, HFD-fed mice exhibited diet duration–dependent delays in TAPF relative to ND-fed mice. Mice on either ND or HFD for 2 and 4 weeks exhibited similar TAPF. However, after 8 weeks of diet, TAPF was significantly (P<0.05) longer for HFD-fed mice compared with that of their ND-fed counterparts (Figure 5F). Again, after acute induction of neutropenia, TAPF was similar for mice fed either an ND or HFD for 8 weeks (Figure 5F).

Finally, although the pseudopod activity levels of neutrophils maintained under static (no flow) conditions after blood draw were unaffected by diet type and duration (Table III in the online-only Data Supplement), HFD altered shear responses (ie, pseudopod retraction) of these cells in a diet duration-dependent fashion. Neutrophils from ND-fed mice for all diet durations exhibited significant (P<0.05) reductions in pseudopod activity in response to in vitro exposure to 5 dyne/cm² for 10 minutes (Table IV in the online-only Data Supplement). Neutrophils from mice subjected to HFD for 2 and 4 weeks exhibited no reductions in pseudopod activity after shear exposure; their shear response indexes approached 0 (Table IV in the online-only Data Supplement). For mice subjected to HFD for 8 weeks, their neutrophils exhibited reversed shear responses with indices that were significantly (P<0.05) <0, that is, pseudopod extension in response to shear (Table IV in the online-only Data Supplement).

Based on Pearson correlation analyses, the neutrophil shear response index, APF, and TAPF from all mice significantly (P<0.05) correlated with serum concentrations of total and free cholesterol (Table). There were also significant (P<0.05) correlations between the neutrophil shear index and either APF or TAPF (Table).

**Discussion**

Pseudopod formation enhances the tumbling of neutrophils in the parabolic velocity field of blood flow in microvessels and promotes their collisions with red blood cells. These collisions displace red blood cells from their axial position and into the peripheral cell-free plasma layer typical of blood flow in microvessels with an adverse effect on blood viscosity. Neutrophil homotypic or heterotypic binding (eg, to platelets) may also amplify these rheological effects.

In contrast, we showed that shear-induced pseudopod retraction minimizes neutrophil tumbling. Conceivably, prestimulated neutrophils in the linear velocity gradient of cone-plate flow retracted their pseudopods, became rounded, and likely reduced their cell–cell collisions (Figure II in the online-only Data Supplement). The neutrophils were likely the major contributors to this effect because of their large numbers in plasma (≈60%–70% of the leukocrit). Monocytes are present in low numbers in plasma (≈5% of the leukocrit) and thus likely had a...
negligible impact. Lymphocytes did not extend pseudopods and thus did not exhibit shear-related retraction (data not shown). Finally, because cell shape changes attributable to bound platelets were likely smaller than those attributable to pseudopods, it was not surprising that platelet binding had no effect.

We reported that membrane cholesterol loading, using CH, dose dependently impairs shear-induced pseudopod retraction. This cholesterol effect on the neutrophils translates to an impact on their flow behavior. Reportedly, 90% of free cholesterol in the cell resides in the peripheral membrane, suggesting that effects of CH likely resulted from its rigidifying actions on this lipid structure. It is possible that CH altered cholesterol distribution among the cytosolic organelles or affected the activity of cell surface proteins because of direct hydrophobic interactions. However, we showed previously that a dose-dependent blockade of neutrophil shear responses by CH results from changes in neutrophil membrane fluidity.

Notably, hypercholesterolemia raises membrane cholesterol content, and reduces membrane fluidity, of leukocytes in blood. Such findings, combined with our data, point to a putative link between blood cholesterol elevations and impaired neutrophil shear regulation. However, the in vitro effects of CH on neutrophil membranes may differ from that due to in vivo blood cholesterol elevations. Low-density lipoprotein particles, the main in vivo cholesterol carriers, may deliver cholesterol into leukocyte membranes less efficiently than CH. Other factors arising from hypercholesterolemia may also alter neutrophil shear sensitivity including changes in plasma composition (eg, inflammatory agonists, proteases) and the activity of other vascular cells. Despite this, the importance of the neutrophil shear response is evident considering that pseudopod formation by flowing leukocytes raises peripheral resistance as previously reported and in line with our own microfluidics data. Consistent with prior studies, we showed that hydrodynamic leukocyte–erythrocyte interactions, and not cell activation alone, are responsible for their impact on flow resistance. The ability of shear to reduce neutrophil pseudopod activity likely minimizes such hydrodynamic interactions and, thus, microvessel flow resistance. Previous in vivo studies have shown that impaired neutrophil pseudopod retraction responses to shear elevate peripheral resistance. But these results point to a passive effect.

Control of neutrophil shear sensitivity may, in fact, contribute to microvascular control of peripheral resistance and tissue blood flow. To explore this possibility, we assessed the neutrophil
impact on RH, which is impaired by hypercholesterolemia.\textsuperscript{6,26} Recently, we developed a procedure\textsuperscript{27} to use DCS to monitor, in real time, rBF in muscles deep (3 mm below the skin) in the thigh of low-density lipoprotein receptor–deficient mice subjected to RH.\textsuperscript{27} DCS has been validated directly in many murine and human tissues against laser Doppler, Doppler ultrasound, Xenon-computed tomography, microsphere velocimetry, power spectral ultrasound, and arterial spin–labeled MRI.\textsuperscript{17,28–30} Moreover, DCS for RH in our mice provided similar blood flow data\textsuperscript{2} to that reported for MNRI-C57BL/6 mice using arterial spin–labeled MRI.\textsuperscript{29} The higher time resolution of DCS, however, allowed us to relate the kinetics of neutrophil shear responses to RH.

Using DCS, we showed that neutrophils (1) play a role in RH and (2) contribute to dysregulated RH during hypercholesterolemia. Notably, the neutropenia data implied that lymphocytes had no impact on RH. A role for monocytes also seemed to be excluded despite reports that their numbers in blood increase during hypercholesterolemia.\textsuperscript{32} Although other Ly6G-positive cells, such as eosinophils and dendritic cells, in blood may affect RH,\textsuperscript{18} their low numbers, relative to neutrophils, likely made their impact small. But cell number is not the main factor because reductions in neutrophil numbers alone did not account for the neutropenic effect on RH in HFD-fed mice. In fact, peripheral resistance is independent of leukocyte concentrations between 3.6×10\textsuperscript{6}/mL and 6.2×10\textsuperscript{6}/mL in blood.\textsuperscript{11}

In addition to neutrophilia, hypercholesterolemia promotes accumulation of activated neutrophils in the microvasculature that perpetuates a chronic inflammatory state and indirectly impacts peripheral resistance via downstream effects on arteriolar endothelium.\textsuperscript{39} Our results substantiate a neutrophil role in hypercholesteremic microvascular pathology.\textsuperscript{10} Specifically, we provide correlative evidence suggesting that in addition to releasing inflammatory agonists, activated neutrophils with impaired shear responses attributable to pathological blood cholesterol elevations also promote microvascular dysfunction by physically disturbing blood flow.

Markedly, time-dependent impairment of neutrophil shear sensitivity long preceded microvascular dysfunction as detected using our novel blood flow indices (ie, APF and T\textsubscript{APF}).

Moreover, the neutrophil impact on RH in our mice largely occurred rapidly after cuff release that tracked with the temporal kinetics of shear-induced pseudopod retraction as defined by our in vitro studies. Thus, APF and T\textsubscript{APF} seem to be sensitive to shear-related neutrophil contributions to RH. In addition, DCS was able to detect these contributions.

However, the link between either APF or T\textsubscript{APF} and neutrophil shear responsiveness reflects a correlative, and not a cause–effect, relationship. Notably, this link was not attributable to differences in baseline pseudopod activity of neutrophils in blood from ND-fed and HFD-fed mice (Table III in the online-only Data Supplement). Although our baseline indices may not represent the instantaneous activity state of neutrophils in vivo, there is no evidence, to our knowledge, that their morphology is altered because of hypercholesterolemia. Finally, the actions of other leukocyte subtypes, platelets, and endothelium on neutrophil activity, for example, via release of cell agonists,\textsuperscript{49} may have contributed to the hypercholesterolemic impact on RH. But despite these possibilities, the neutrophil seems to be the key player, as verified by our neutropenia results.

Moreover, the correlation between shear sensitivity and RH did not account for neutrophil adhesion in the microcirculation, which can dramatically raise hemodynamic resistance by reducing microvascular radii.\textsuperscript{11} Shear stress is antiadhesive for neutrophils by promoting cleavage of cell surface CD18 integrins.\textsuperscript{30,42} Notably, cholesterol influences the regulation of CD18-related neutrophil adhesiveness.\textsuperscript{42,43} Its enrichment in the cell membrane raises CD18 surface levels and neutrophil adhesion.\textsuperscript{52,45} Thus, the major impact of hypercholesterolemia on RH may, in fact, be attributable to its effects on shear-sensitive CD18 proteolysis. Despite this, our data are still the first to implicate neutrophil shear sensitivity in RH.

Considering that microvascular dysfunction forecasts hypercholesterolemic vasculopathy,\textsuperscript{4,5} impaired neutrophil shear sensitivity upstream of dysregulated RH may be an early symptom of the harmful impact of high blood cholesterol. Thus, ex vivo blood cell measures and in vivo tissue blood flow indices that account for neutrophil shear sensitivity may be prognostic, and not just diagnostic, of hypercholesterolemia-related pathobiology. Our results also point to a potential strategy to target a source of microvascular dysfunction attributable to hypercholesterolemia. Specifically, because acutely fluidizing the membranes of neutrophils counteracts the effects of excess cholesterol on their shear responsiveness,\textsuperscript{15} membrane fluidizers may be used to ameliorate microvascular dysfunction attributable to hypercholesterolemia while administering cholesterol-lowering drugs during the long term. The potential benefit of membrane fluidizers on hypercholesterolemic microvasculature has been supported by evidence from other investigators.\textsuperscript{41} In these ways, the insight revealed by this study may serve as the basis for the design of new clinical approaches focused on the pathobiology, and not just on cholesterol levels, something that has come into question as the most effective strategy to treat hypercholesterolemic vascular disease.

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Disclosures

None.

References

Neutrophil shear responses and tissue blood flow

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Neutrophils have largely been neglected in hypercholesterolemia-related pathobiology. We integrated the use of cell mechanics with microfluidics methodologies to link cholesterol-related control of neutrophil pseudopod activity by shear stress to microvessel flow resistance. Moreover, using novel measures derived from a noninvasive, diffuse correlation spectroscopic technique, we revealed a first correlation linking hypercholesterolemic microvascular dysregulation, neutrophils and their impaired shear sensitivity. These results provide new insight regarding the link between hypercholesterolemia and microvascular dysfunction. Microvascular dysfunction long precedes, and is proposed to forecast, lethal hypercholesterolemia vasculopathy. In light of this, our study suggests that impaired neutrophil shear sensitivity is one of the earliest symptoms of the hypercholesterolemic impact on the microcirculation. As such, our results may serve as the basis for novel diagnostic strategies and therapeutic (eg, membrane fluidizing adjuvant) approaches that target an early symptom (eg, impaired neutrophil shear regulation) of hypercholesterolemic pathobiology other than cholesterol levels.
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THE SHEAR-SENSITIVE REGULATION OF NEUTROPHIL FLOW BEHAVIOR AND ITS POTENTIAL IMPACT ON MICROCIRCULATORY BLOOD FLOW DYSREGULATION IN HYPERCHOLESTEROLEMIA

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SUPPLEMENTAL FIGURE AND TABLE

Supplemental Figure I. Representative images of pseudopod-positive (+) and -negative (-) neutrophils. Nuclei were stained with DAPI (green).
Supplemental Figure I. The predicted tumbling behavior of neutrophils in the linear flow field of a cone-plate rheometer during viscosity measurements. Viscosities of neutrophil suspensions may be influenced by pseudopod projection upon cell activation which enhances neutrophil tumbling in the flow field and thus increases stochastic (bumping) interactions (i.e., collisions) between cells. Shear-induced deactivation of cells is predicted to promote smoother flow of cells within the cone-plate flow field leading to reduced viscosities.

Supplemental Table I. Cholesterol concentrations in plasma of LDLr−/− mice.

<table>
<thead>
<tr>
<th>Cholesterol Type</th>
<th>Diet</th>
<th>Cholesterol Concentrations (mg/dl)</th>
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<tbody>
<tr>
<td></td>
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<td>2 weeks</td>
</tr>
<tr>
<td>Free</td>
<td>ND</td>
<td>70 ± 6</td>
</tr>
<tr>
<td></td>
<td>HFD</td>
<td>335 ± 18 *</td>
</tr>
<tr>
<td>Total</td>
<td>ND</td>
<td>251 ± 11</td>
</tr>
<tr>
<td></td>
<td>HFD</td>
<td>886 ± 49 *</td>
</tr>
</tbody>
</table>

Data are mean ± SEM from n = 7 experiments using regular mice and n = 6 experiments using neutropenia mice. *p<0.05 compared to ND; †p<0.01 compared to 2 weeks; Student’s t-test with Bonferroni’s adjustment.
### Supplemental Table II: rBF during 5 minutes of cuff occlusion.

<table>
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<tr>
<th>Diet</th>
<th>2 weeks</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>8 weeks Neutropenia</th>
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</thead>
<tbody>
<tr>
<td>ND</td>
<td>7.46 ± 0.81</td>
<td>5.94 ± 1.34</td>
<td>5.94 ± 1.62</td>
<td>4.59 ± 0.48</td>
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<tr>
<td>HFD</td>
<td>6.70 ± 1.78</td>
<td>5.22 ± 0.49</td>
<td>3.58 ± 0.65</td>
<td>8.87 ± 0.81</td>
</tr>
</tbody>
</table>

Data are mean ± SEM from n = 7 experiments using regular mice and n = 6 experiments using neutropenia mice. No significant differences were detected using 2-way ANOVA.

### Supplemental Table III. The baseline pseudopod activity level of neutrophils from LDLr<sup>-/-</sup> mice under in vitro no-flow condition.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Percentage of Pseudopod-Positive Neutrophils (%)</th>
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<tbody>
<tr>
<td></td>
<td>2 weeks</td>
</tr>
<tr>
<td>ND</td>
<td>37 ± 8</td>
</tr>
<tr>
<td>HFD</td>
<td>26 ± 6</td>
</tr>
</tbody>
</table>

Data are mean ± SEM from n = 7 experiments. No significant differences were detected using 2-way ANOVA.
### Supplemental Table IV. The shear-induced pseudopod retraction response by neutrophils from LDLr⁻/⁻ mice under in vitro cone-plate shear flow.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Shear Response (%)</th>
<th>2 weeks</th>
<th>4 weeks</th>
<th>8 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND</td>
<td>47.3 ± 3.8 #</td>
<td>42.5 ± 10.3 #</td>
<td>47.2 ± 5.5 #</td>
<td></td>
</tr>
<tr>
<td>HFD</td>
<td>-2.4 ± 33.1</td>
<td>-6.2 ± 17.4 *</td>
<td>-98.5 ± 38.2 **</td>
<td></td>
</tr>
</tbody>
</table>

Data are mean ± SEM from n = 7 experiments. *p<0.05; 2-way ANOVA detected diet had effects, then Student’s t-test was performed between ND and HFD at each time point. #p<0.05 compared to a value of 0 using one-sample t-test; positive indexes mean shear reduces pseudopod activity while negative indexes mean shear increases pseudopod activity.
THE SHEAR-SENSITIVE REGULATION OF NEUTROPHIL FLOW BEHAVIOR AND ITS POTENTIAL IMPACT ON MICROVASCULAR BLOOD FLOW DYSREGULATION IN HYPERCHOLESTEROLEMIA

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MATERIALS AND METHODS

Human peripheral blood neutrophils  
Fresh human peripheral blood was harvested from normal volunteers into K2-EDTA-coated vacutainers (Becton-Dickinson) using informed consent and standard venipuncture procedures that were approved by the Institutional Review Board at the University of Kentucky.  

For some experiments, leukocyte-enriched plasma (containing neutrophils, monocytes, lymphocytes, platelets and sporadic RBCs) was harvested after 1xg sedimentation at room temperature for 45 minutes and subsequently diluted in Hank’s balanced salt solution with Ca2+ and Mg2+ (HBSS; 1:10 v/v; Invitrogen). Addition of Ca2+ and Mg2+ ions has been shown to restore the adhesive function of platelets that have been exposed to EDTA.1  

For some experiments, human neutrophils were purified from whole blood using two-step Histopaque-Percoll gradient centrifugation following established procedures.3 These cells were subsequently resuspended in phosphate-buffered saline (PBS; Mediatech) at 2x10⁷ cells/ml. The resultant purified cell suspensions contained >90% neutrophils (data not shown).

Chemical treatments of human neutrophils  
Experiments were designed to test the ability of fluid shear stress to restrict/reverse mild cell activation, for example, due to fluid stasis, blood isolation, or stimulation with “low” doses of cell agonists such as f-Met-Leu-Phe (fMLP; <10⁻⁶ M) and platelet-activating factor (<10⁻⁷ M).4 We treated human leukocytes with 10 nM fMLP (Sigma Aldrich) for 10 minutes to raise the baseline activity of cells to permit elucidation of the effects of neutrophil shear responses on viscosity with more sensitivity. We also used E64 to enhance neutrophil-platelet binding during viscometry to levels that were unlikely to be seen in the physiologic circulation except under severe pathobiology.5 In this case, E64 (28 µM; MP Biochemicals)7 was added to the suspending medium during the 10 minute cell pre-stimulation period and subsequent viscometric analyses. To enhance membrane cholesterol in vitro, cells were incubated with 2 – 10 µg/ml cholesterol:methyl-β-cyclodextran complexes (CH; Sigma Aldrich) for 15 minutes prior to fMLP stimulation.8 Cell viability was assessed using a Live/Dead Double Staining Kit (Calbiochem) and remained unchanged at >90% after all incubations (data not shown).
Viscometric analyses of human leukocyte suspensions

Suspensions of leukocytes were subjected to a constant shear rate (450 second⁻¹) for 10 minutes using a computer-interfaced DV-II+PRO digital cone-plate viscometer (Brookfield). The shear rate used for the present study is within the physiologic range (250 – 2000 second⁻¹) reported for the microcirculation, particularly the pre-capillary arterioles and post-capillary venules. During flow exposure, the viscosity of cell suspensions was recorded at 1 minute intervals using Wingather 32 software (Brookfield). The temperature of cell suspensions was maintained at room temperature throughout the whole procedure using a water-jacketed cooling system incorporated into the lower plate of the cone-plate system.

Analyses of shear-induced human neutrophil deactivation

Suspensions of fMLP-stimulated leukocytes were exposed to 5 dyn/cm² for 0 – 10 minutes in a custom cone-plate rheometer. After experiments, cells were fixed immediately in 1% paraformaldehyde (Electron Microscopy Sciences) for at least 10 minutes.

For analysis of pseudopod activity, fixed cells were permeabilized with 0.01% Triton X-100 in PBS for 1 minute and labeled with 0.2 µg/ml 4',6-diamidino-2-phenylindole (DAPI; MP Biochemicals). Neutrophils with multi-lobed nuclei were observed with an IX-70 inverted fluorescence microscope (Olympus) at a 400X magnification and ultraviolet illumination (excitation/emission wavelength: 358/461 nm). For each sample, a minimum of 30 cell morphologies were visually assessed using brightfield imaging. The percentage of cells within a population that displayed at least 1 cellular projection was used to quantify the cell activation level of the neutrophil suspensions.

For neutrophil-platelet binding studies, fixed cells were labeled with antibodies against platelet-specific CD41 (1:20 v/v; FITC conjugated clone VIPL3; Cat # MHCD4101; Invitrogen) and leukocyte-specific CD45 (1:20 v/v; PE-Cy™7 conjugated clone HI30; Cat # 560915; Becton-Dickinson) in PBS at 4 ℃ in the dark for 30 minutes. Stained cells were rinsed and analyzed for surface-bound antibodies using an LSR II flow cytometer (Becton-Dickinson) interfaced with Becton-Dickinson FACsDiva software. Neutrophils were gated using a combination of forward (FSC) and side scatter (SSC) and positive anti-CD45 fluorescence (i.e., CD45+). For each sample, ~10,000 neutrophils were analyzed. Non-specific binding was assessed using isotype-matched mouse IgG1 (1:20 v/v; Alexa Fluor®488 conjugates; Cat # MG120; Invitrogen). The percentage of CD41+ neutrophils was quantified and used as a measure of neutrophil-platelet binding.

Impact of neutrophil activation on microchannel flow resistance

In order to assess the potential impact of neutrophil pseudopod activity on microvessel (i.e., arteriolar/venular) flow resistance, a polydimethylsiloxane microfluidic chamber with a single rectangular microchannel (w: 500 µm; h: 50 µm; l: 20 mm) was fabricated as reported previously. At microcirculatory flow rates (i.e., ~0.03 – 2.5 ml/h), the Reynolds Number for flow through the custom microchannel is extremely small (<<1) ensuring laminar flow conditions. To measure pressure differences across the microchannel, the infusion port of microfluidic chamber was connected to a pre-calibrated Statham pressure transducer via a piece of polyvinyl chloride (PVC) tubing (inner diameter: 0.010”) with the outlet open to atmosphere (Methods Figure I). A pressure transducer was interfaced to a carrier demodulator (Validyne) and a data acquisition device (NI USB-6008; National Instruments).

Purified neutrophils (1x10⁶ cells/ml) were stimulated with 10 nM fMLP for 5 minutes and fixed with 1% paraformaldehyde. Cells without stimulation served as controls. To assess the effects of neutrophil-RBC interactions on flow resistance, autologous RBCs were recombined with neutrophil suspensions to a hematocrit of 10%. Addition of RBCs was conducted so as to maintain neutrophil densities similar to those used for analyses of purified cell suspensions.
Moreover, this approach was estimated to reflect a physiologic level of neutrophils present in the blood passing through the non-capillary microvasculature.

Cell suspensions were driven through the microfluidic chamber at 1 ml/h for 5 min using a Harvard Apparatus syringe pump (model 2000; Methods Figure I). Prior to their entry into the microchannel, cell suspensions were maintained under constant agitation using a stir plate in conjunction with a magnetic stir bar placed inside the syringe containing the perfusate. The infusing pressure (relative to atmosphere) was recorded in real-time by LabVIEW SignalExpress (National Instruments). The instantaneous pressure readings ($P_t$) over the last 2-min were meaned (i.e., $\bar{P}$) and used to calculate flow resistance ($R$) using the following equation:

$$ R = \frac{P_t - P_{atm}}{Q} $$

where $Q$ is the volumetric flow rate of the syringe pump.

**Quantification of RH in hypercholesterolemia mice**

To examine the role of cholesterol-dependent neutrophil shear mechanotransduction in microvascular blood flow regulation, the RH response of skeletal muscles was tested in a hypercholesterolemia model. All animal handling procedures used for the present study were approved by the University of Kentucky Institutional Animal Care and Use Committee.

To induce hypercholesterolemia, LDLr<sup>−/−</sup> mice (male; 8-week age; B6.129S7-Ldlr<sup>tm1Her</sup>, Cat # 2207; the Jackson Laboratory) were fed a diet enriched in saturated fat (HFD) (21% wt/wt fat and 0.15% wt/wt cholesterol; Harlan Teklad; Cat # TD88137) ad libitum for up to 8 weeks as described. Age-matched LDLr<sup>−/−</sup> mice fed a normal laboratory diet (ND) served as controls. To confirm the neutrophil contribution to RH, acute neutropenia was induced in LDLr<sup>−/−</sup> mice that had been placed on an ND or HFD for 8 weeks by i.p. injection of 1.0 mg of anti-Ly6G (1A8; Cat # BE0075-1; BioXCell) 36 hours prior to use. Neutrophils in these antibody-treated animals accounted for $<$1.0% of total leukocytes as compared to around 10% in regular mice as assessed by flow cytometry (data not shown).

At selected time points (i.e., 2, 4, and 8 weeks of diet), we used NIR-DCS to non-invasively record the tissue blood flow responses in thigh muscles of the LDLr<sup>−/−</sup> mice subjected to 5 minutes of cuff occlusion as reported. NIR-DCS is based on an approximation for light scattering over long distances as a diffusive process. NIR light ($\lambda=785$ nm) transmitted through...
an optical fiber is launched into tissue. A single-mode detector fiber placed on the skin at a known distance from the source fiber detects light intensity fluctuations within a single speckle area of tissue. From these, a light intensity autocorrelation function satisfying the correlation diffusion equation in highly light-scattering media\(^\text{12}\) is computed. The solution for the correlation diffusion equation, which describes speckle fluctuations of diffused light, extracts a blood flow index (BFI) based on the red blood cell motion and tissue blood volume fraction. rBF is calculated by normalizing BFI to its baseline value before arterial occlusion.

NIR-DCS measurements were conducted on mouse hindlimbs (Methods Figure II) using our unique set up.\(^\text{11}\) Briefly, mice in a prone position were anesthetized by inhalation of 1% isoflurane (Butler Schein) with body temperature maintained at 37 °C on a heating pad. A NIR-DCS probe (i.e., a foam pad) housing a NIR light source and a detector separated by a distance of 6 mm was super-glued to the shaven skin overlying the posterior thigh muscles of the

hindlimb. The 6-mm separation distance ensured detection of relative changes of blood flow (rBF) 3 mm under the skin surface deep within the muscle tissue (Methods Figure II).\(^\text{13, 14}\) Upstream of the probe, a piece of PVC tubing (outer diameter: 0.05”) was loosely wrapped into a noose (i.e., cuff) around the proximal end of the limb without restricting blood flow, and rBF was measured with the NIR-DCS device for 5 minutes to acquire a baseline. Cuff occlusion was then applied to the thigh by tightening the PVC tubing noose until rBF was reduced to less than 10% of its baseline levels and maintained for 5 minutes. To release the occluding cuff, the PVC tubing noose was severed using a high temperature surgical cautery pen. Real-time rBF was recorded until it returned to baseline (pre-occlusion) levels.

**Analyses of neutrophil shear mechanotransduction in murine blood**

Immediately after NIR-DCS tissue blood flow measurements, mouse blood (~500 µl) was harvested into a 1-ml syringe containing EDTA (20 µl of 0.2 M) by thoracotomy and cardiac puncture as reported.\(^\text{6}\) Within 3 hours of blood collection, aliquots of whole blood were diluted 1:20 (v/v) with HBSS and subjected to cone-plate shear exposure (5 dyn/cm\(^2\); 10 min). The
shear-induced pseudopod retraction responses of the neutrophils were calculated as described using the following equation:

Shear Response (%) = \frac{\% \text{ cells with pseudopods}_{\text{static}} - \% \text{ cells with pseudopods}_{\text{shear}}}{\% \text{ cells with pseudopods}_{\text{static}}}.

Murine plasma cholesterol quantification
Murine plasma was extracted from whole blood by centrifugation under 1000xg at 4 °C for 5 minutes. Concentrations of free and total cholesterol in plasma samples were determined with a spectrophotometer (BioTek; µQuant) using commercially-available Free Cholesterol E (Wako) and Infinity Cholesterol Reagent (Sigma Aldrich) kits, respectively.

Statistics
Statistical analyses were performed with SigmaPlot 12.3 (Systat Software Inc., San Jose, CA, USA). Parametric data were expressed as mean ± SEM; differences between means were assessed by one-way ANOVA (with/without repeated measures) or Student’s t-test. Normalized data expressed as Shear Response indices were compared to a value of 0 using one-sample t-test. Dunnett’s or Bonferroni’s method was used as a post hoc test when appropriate. Non-parametric data were displayed as box-and-whisker plots; differences between medians were assessed by Mann-Whitney U tests. Pearson correlation analyses were conducted to assess the relationships between key parameters of interest (e.g., plasma cholesterol levels, neutrophil shear responses, APF, and T_{APF}).

REFERENCES

