UltraRapid Communication

Mechanisms for Regulation of Fluid Shear Stress Response in Circulating Leukocytes

Shunichi Fukuda, Takanori Yasu, Dan N. Predescu, Geert W. Schmid-Schönbein

Abstract—We have shown that leukocytes retract their pseudopods and detach from substrates after exposure to physiological fluid shear stresses (≈1.5 dyn/cm²). In inflammation, however, pseudopod projection during spreading and firm adhesion on endothelium is observed even in microvessels with normal blood flow and fluid shear stresses. Thus, we examined mechanisms that may serve to regulate the shear stress response of circulating leukocytes. In the presence of inflammatory mediators (platelet-activating factor [PAF] f-met-leu-phe), a subgroup of cells ceases to respond to shear stress. cGMP analogs and nitric oxide (NO) donors enhance the shear stress response and reverse the inhibitory effect of inflammatory mediators on the shear stress response, whereas depletion of cGMP leads to cessation of the shear stress response even in unstimulated leukocytes. The ability of cGMP to enhance the shear stress response is not associated with CD18 expression, because cGMP has no effect on CD18 expression in response to shear stress. The shear stress response of leukocytes in endothelial nitric oxide synthase (−/−) mice, in which NO level in blood is decreased, is attenuated compared with that in wild-type mice. In rat mesentery venules stimulated by PAF under normal blood flow, a cGMP analog diminishes pseudopod projection of leukocytes, whereas inhibition of NO leads to enhanced pseudopod projection and spreading. The evidence suggests that inflammatory mediators suppress the shear stress response of leukocytes leading to spreading even under normal physiological shear stress, whereas cGMP may serve to maintain shear stress response even in inflammation. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2000;86:e13-e18.)

Key Words: leukocyte ■ microcirculation ■ shear stress ■ nitric oxide ■ cGMP

There is now evidence that all cells in the circulation respond to physiological shear stress, including endothelial cells, 1 erythrocytes, 2 platelets, 3 and leukocytes. 4 Human leukocytes (neutrophils, monocytes, and lymphocytes) respond to fluid shear stress by retraction of pseudopods (lamellipodia, filopodia, and other forms) and membrane detachment. 4 The shear stress response of leukocytes serves as the key mechanism to maintain circulatory leukocytes in a spherical shape without pseudopod formation. In the presence of pseudopods, leukocytes become trapped in capillaries with high probability. 5 Pseudopod retraction is a requirement for normal passage of circulating leukocytes through the microcirculation.

In inflammation, however, pseudopod projection (change of the cell shape from a spherical shape) of adherent leukocytes is a requirement for migration across the endothelium into the tissue. The adhesion is readily observed even in venules that have essentially normal blood flow. 6 Thus, there may exist a fundamental regulating mechanism for the leukocyte shear stress response in inflammation. Using in vitro experiments on human, rat, and mouse leukocytes and in vivo observations in the rat mesentery, we provide evidence in the present study that inflammatory mediators suppress pseudopod retraction by fluid shear stress, and that the shear stress response of leukocyte plays an inhibitory role in inflammation, which is enhanced by nitric oxide (NO) via cGMP.

Materials and Methods

The study protocol was reviewed and approved by the University of California San Diego Human and Animal Subject Committee.

In Vitro Experiments Using Human Blood

After red cell sedimentation, the supernatant of leukocytes collected from volunteers was resuspended in Plasma-Lyte (Baxter Health Care) with 2.5 mmol/L Ca²⁺. The cell suspension (100 μL) was deposited into a chamber on an inverted microscope (Leitz). Micropipettes of 4 to 6 μm in diameter were connected to a reservoir with hydrodynamic pressure adjustment and were positioned above a neutrophil on the glass so that fluid flow could be applied over the cell surface. The magnitude of the fluid stress on the cell surface was numerically computed. 4 The maximum length across the cell and pseudopod, L, was measured and normalized by the diameter of the cell, Loo, without pseudopod length (see Figure 1). The cell suspension 2 hours after the blood collection was also placed on a glass without shear stress for 30 minutes, and L in 100 cells of each group was measured.

Received December 6, 1999; accepted December 6, 1999.

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In Vitro Studies in a Cone-and-Plate Shear Field

Because procedures for isolation of living leukocytes significantly affect the shear stress response (data not shown), unseparated whole blood was used. Methylene blue, 8-bromoguanosine 3'-5'-cyclic monophosphate (8-Br-cGMP), or 0.9% saline as control were applied to each group 30 minutes after the blood collection from Wistar rats and endothelial nitric oxide synthase (eNOS) (−/−) and wild-type mice (C57BL/6J-NOS−/−, Jackson Laboratory, Bar Harbor, Maine). The whole blood (0.3 mL) was sheared in a cone-and-plate device at a level of 5.0 dyn/cm² for 10 minutes and fixed with 2% glutaraldehyde. To measure CD18 expression, the cells were fixed in 0.4% paraformaldehyde. Unsheared control samples were also fixed at the same time in the same way. The number of leukocytes with pseudopods stained by 0.02% crystal violet was counted by light microscopy. To determine CD18 membrane expression, FITC-labeled monoclonal antibody against rat CD18 (PharMingen) or rat IgG isotype (PharMingen) was applied to each blood group for 30 minutes. Red cells were removed by FACS Lysing Solution (Becton Dickinson). CD18 expression was measured with a flow cytometer (Becton Dickinson FACS analyzer).

TABLE 1. Effects of cGMP in the Absence of Shear Stress on the Projected Cell Length

<table>
<thead>
<tr>
<th></th>
<th>Length, μm</th>
<th>Significance</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>12.6±2.8</td>
<td>...</td>
</tr>
<tr>
<td>8-Br-cGMP</td>
<td>12.5±2.3</td>
<td>...</td>
</tr>
<tr>
<td>PAF</td>
<td>15.6±3.4</td>
<td>*</td>
</tr>
<tr>
<td>PAF+8-Br-cGMP</td>
<td>13.5±2.7</td>
<td>†</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>17.8±3.6</td>
<td>†, †</td>
</tr>
</tbody>
</table>

The lengths of 100 projected cells 30 minutes after application of agents (10 μmol/L PAF, 100 μmol/L 8-Br-cGMP, and 100 μmol/L methylene blue) in the absence of flow were measured in each group. All results are expressed as mean±SD. *P<0.05 vs control; †P<0.05 vs PAF.

NO Metabolites Measurement in Mice Blood

After centrifugation of mouse blood, NO metabolites (nitrite/nitrate) in serum were measured using an NO analyzer (ENO-20; Eicom Corp).

In Vivo Observation of Pseudopod Formation

The mesenteric microcirculation was visualized with an intravital microscope (Leitz). To reduce the shear stress in microvessels to near zero, postcapillary venules were occluded for 3 minutes with a micropipette mounted on a micromanipulator. The pseudopod formation was observed during flow occlusion and after flow restoration for 2 minutes. Except for controls, the superfusion contained 10⁻⁴ mol/L platelet-activating factor (PAF). For measurement of the shear rate in venules, 0.1 mL of erythrocytes from donor rats labeled with fluorescent dye PKH-26 (Zynaxis Cell Science) was intravenously injected. The velocities of 50 labeled erythrocytes in each venule were measured using an intravital fluorescence microscope with a SIT camera (Dage-MTI). The shear rate on the endothelium in each venule was estimated as 4×mean velocity/terminal radius of the vessel.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

In Vitro Observations on Adherent Leukocytes

Application of shear stresses (≈1.5 dyn/cm²) on adherent leukocytes on a glass surface caused instant pseudopod retraction irrespective of the particular shape of the pseudopod (Figures 1A through 1D and 2A). No pseudopod projection occurred during shear stress application (Figure 2A). This response was observed in all fresh human leukocytes (50 of 50 tested cells).

Inflammatory Stimulators and the Shear Stress Response of Adherent Neutrophils In Vitro

The inflammatory stimulators PAF and formyl-methionyl-leucyl-phenylalanine (FMLP) suppressed the shear stress response of neutrophils in a dose-dependent manner (Figures 2B and 2C). Cells that showed a reduced response to fluid shear stress tended to spread out on the substrate and exhibited reduced migration.

cGMP and the Shear Stress Response of Adherent Neutrophils In Vitro

The soluble guanylate cyclase inhibitor methylene blue served to completely block the shear stress response (Figures...
and also led to enhanced spreading of the cells (Figures 1E through 1H). In contrast, pseudopod retraction velocity was enhanced by the cGMP analog 8-Br-cGMP, at 1.5 dyn/cm² (Figure 2D). The soluble guanylate cyclase blocker LY83583 (100 \( \mu \text{mol/L} \)) also inhibited the shear stress response at 1.5 dyn/cm² (results not shown). In the absence of fluid shear stress and without any inflammatory stimulator, 8-Br-cGMP had no effect on the average cell length projected on a glass surface, whereas the average length of the cells treated with PAF was reduced by 8-Br-cGMP (Table 1).

**Effect of cGMP and NO Donors on the Shear Stress Response of Stimulated Leukocytes In Vitro**

In the presence of PAF, \( L/L_0 \) of leukocytes treated with 8-Br-cGMP was significantly lower during fluid shear application (Figure 2B). The suppression of the fluid stress response by PAF and FMLP could be attenuated in a dose-dependent manner by the cGMP analogs 8-Br-cGMP and dibutyryl-cGMP (Figure 3). The NO donors \( S^-\text{nitroso-acetyl penicillamine} \) (SNAP) and 3-morpholinosydnonimine (SIN-1) also enhanced the response to shear stress of neutrophils stimulated by PAF or FMLP. Superoxide dismutase (SOD) by itself did not enhance the shear stress response. The effect of NO donors on the fluid stress response was, however, mildly enhanced by SOD. NOS inhibitors, such as \( N^-\text{G}-\text{methyl- L -arginine} \) (L-MNA; 1 mmol/L) and \( N^-\text{G}-\text{nitro- L -arginine methyl ester} \) (L-NAME; 1 mmol/L), did not affect the shear stress response in vitro in both the absence (data not shown) and presence of PAF or FMLP (Figure 3).

**Effect of cGMP and NO Donors on the Shear Stress Response of Suspended Leukocytes In Vitro**

Because the experimental observations on single cells depend on their adhesion to a substrate, we introduced a technique that permits the study of leukocytes in free suspension under well-controlled shear stresses. In line with the experiments on 2B and 2C and also led to enhanced spreading of the cells (Figures 1E through 1H). In contrast, pseudopod retraction velocity was enhanced by the cGMP analog 8-Br-cGMP, at 1.5 dyn/cm² (Figure 2D). The soluble guanylate cyclase blocker LY83583 (100 \( \mu \text{mol/L} \)) also inhibited the shear stress response at 1.5 dyn/cm² (results not shown). In the absence of fluid shear stress and without any inflammatory stimulator, 8-Br-cGMP had no effect on the average cell length projected on a glass surface, whereas the average length of the cells treated with PAF was reduced by 8-Br-cGMP (Table 1).

**Figure 2.** A and B, Time course of human leukocytes spreading before and after application of shear stress for 3 minutes with 10 \( \mu \text{mol/L} \) PAF, 100 \( \mu \text{mol/L} \) 8-Br-cGMP, or 100 \( \mu \text{mol/L} \) methylene blue. \( L \) indicates the maximum cell length; \( L_0 \), the diameter in a spherical state. \( n=6 \) in each group. \( L/L_0 \) during shear application in the control and PAF+8-Br-cGMP groups was significantly lower than before shear application. *\( P<0.05 \) vs no shear stress (A); **\( P<0.05 \) vs PAF (B). C, Effects of PAF (\( \bullet \)) and FMLP (\( \square \)) on the neutrophil response to shear stress. \( n=20 \) in each group. Responding leukocytes are defined as the cells in which >20% reduction of \( L/L_0 \) is observed during shear stress. D, Pseudopod retraction velocity in response to shear stress with

**Figure 3.** Effects of 10 and 100 \( \mu \text{mol/L} \) 8-Br-cGMP, 10 and 100 \( \mu \text{mol/L} \) dibutyryl-cGMP, 410 U/mL SOD, 1 mmol/L L-NAME, 1 mmol/L L-MNA, 100 \( \mu \text{mol/L} \) SNAP, and 100 \( \mu \text{mol/L} \) SIN-1 on the shear stress response of neutrophils treated with 10 \( \mu \text{mol/L} \) PAF (\( \bullet \)) or FMLP (\( \square \)). \( n=10 \) to 20 in each group. Responding leukocytes are defined as the cells in which >20% reduction of \( L/L_0 \) is observed during shear stress application. *\( P<0.05 \) vs control.
adherent cells, the number of leukocytes with pseudopod formation without treatment was significantly decreased by application of shear stress (5.0 dyn/cm²) with the cone-and-plate shear device (Figure 4A). 8-Br-cGMP enhanced pseudopod retraction of circulating cells in response to shear stress, whereas methylene blue significantly suppressed the response (Figure 4A).

In contrast, at a shear stress of 5 dyn/cm², neither 8-Br-cGMP nor methylene blue had any significant effect on the expression of CD18 of leukocytes (Figure 4B), although cGMP had a tendency to reduce CD18 expression in the absence of shear stress, and methylene blue tended to increase CD18 expression (Figure 4B). Shear stress served to reduce the CD18 expression with or without cGMP present (Figure 4B).

**Shear Stress Response of Leukocytes in eNOS Knockout Mouse**

Although the number of leukocytes with pseudopod formation in both eNOS (−/−) and wild-type mice was decreased by application of shear stress (5.0 dyn/cm²) with the cone-and-plate shear device, leukocytes of eNOS (−/−) mice responded less to shear stress compared with wild types (Figure 4C). Plasma NO metabolite levels in eNOS knockout mice were significantly lower than in wild types (Table 2).

**Shear Stress Response In Vivo**

Nonstimulated leukocytes projected pseudopods during vessel obstruction in every direction. During obstruction, 5 of 8 adhered leukocytes on the vessel wall projected pseudopods; 3 of 8 did not. Adherent leukocytes retracted their pseudopods immediately after restoration of fluid stress and were washed away in the bloodstream once they reached an almost spherical shape (L/L₀ ≈ 1) (Figures 5A through 5C). Nonadherent leukocytes were washed away in the bloodstream immediately after flow restoration.

In contrast, in the presence of 10⁻³ mol/L PAF without shear stress, the majority of pseudopods were projected over the endothelial cells, fewer pseudopods were pointing into the vessel lumen, and all leukocytes (8 of 8 cells) adhered to and crawled on the vessel wall during flow obstruction. Pseudopod retraction in response to fluid stress could be suppressed by application of PAF, which caused firm leukocyte adhesion (3 of 8). A combination of NOS inhibition by L-MNA (2.5 mg/kg) with PAF application led to diminished pseudopod retraction compared with the levels with PAF without L-MNA after flow restoration, and, consequently, to an increased count of adherent (3 of 8) as well as migrating (3 of 8) leukocytes (Figures 5D through 5F). 8-Br-cGMP (0.45 mg/kg) diminished the ability of PAF to suppress the shear stress response in terms of pseudopod projection and reversed the ability of L-MNA to further block the shear stress response. In both PAF with 8-Br-cGMP and PAF with L-MNA and 8-Br-cGMP groups, all leukocytes retracted their pseudopods and were washed away in the bloodstream within 15 seconds after flow restoration. l-Arginine (300 mg/kg) also reversed the effect of L-MNA on the shear stress reaction in the presence of PAF. There are no significant differences in microvessel wall shear rates between groups (669.0 ± 162.8 1/sec).

**Discussion**

The present study provides evidence that the response of leukocytes to shear stress, in terms of pseudopod retraction, is
dopod retraction in response to shear stress. cGMP may be one of the requirements for leukocyte pseudopod retraction. Inflammatory stimulators can be attenuated in a dose-dependent manner by cGMP, NO, and inflammatory stimulators in vivo, we also examined pseudopod formation by leukocytes in rat mesentery venules after reduction and restoration of physiological shear stresses. Nonstimulated leukocytes project pseudopods during vessel obstruction but retracted them immediately after restoration of fluid stress. After retraction of the pseudopods, the leukocytes were washed away from the endothelium by the bloodstream (Figures 5A through 5C). Pseudopod retraction in response to fluid stress is reduced in a dose-dependent manner (Figures 2B and 2C). Moreover, the suppressors of soluble guanylate cyclase inhibit the response (Figures 2B, 2C, 2D, and 3). A and D. Spherical shape of leukocytes without pseudopods immediately after obstruction. B and E. Leukocyte pseudopod projection (arrows) and spreading on the vessel wall during zero flow period. C and F. Leukocyte shapes after flow restoration. The nonstimulated leukocyte retracts its pseudopods and assumes a spherical shape (C), whereas the stimulated cell continues to spread on the endothelium without response to fluid stress 2 minutes after flow restoration (F) (small arrows). Large arrows indicate direction of blood flow.

Figure 5. Time course of pseudopod formation of leukocytes without (A through C) or with (D through F) application of 10^-4 mol/L PAF and 2.5 mg/kg L-MNA in rat mesentery venules. A and D. Spherical shape of leukocytes without pseudopods immediately after obstruction. B and E. Leukocyte pseudopod projection (arrows) and spreading on the vessel wall during zero flow period. C and F. Leukocyte shapes after flow restoration. The nonstimulated leukocyte retracts its pseudopods and assumes a spherical shape (C), whereas the stimulated cell continues to spread on the endothelium without response to fluid stress 2 minutes after flow restoration (F) (small arrows). Large arrows indicate direction of blood flow.

suppressed at elevated concentrations of inflammatory mediators. In contrast, the shear stress response is enhanced by the cGMP analogs, whereas the blockers of soluble guanylate cyclase inhibit the response (Figures 2B, 2C, 2D, 3, and 4A). cGMP may be one of the requirements for leukocyte pseudopod retraction in response to shear stress.

Although pseudopods on circulating leukocytes play an important role during trapping in capillaries, they are essential for migration and phagocytosis. After stimulation with inflammatory mediators, the number of leukocytes that retracted their pseudopods after exposure to fluid shear stress is reduced in a dose-dependent manner (Figures 2B and 2C). Moreover, the suppression of the fluid stress response by inflammatory stimulators can be attenuated in a dose-dependent manner by cGMP analogs (Figures 2B and 3). The evidence thus suggests that cGMP and inflammatory stimulators have the opposite effect of each other on the shear stress response and on pseudopod formation during inflammation. In the presence of fluid shear stress, cGMP increases the sensitivity of leukocytes to shear stress and promotes pseudopod retraction, whereas inflammatory stimulators attenuate the shear stress response by shifting from pseudopod retraction to pseudopod projection. In inflammation, both cGMP and inflammatory stimulators may regulate the shear stress response of neutrophils. The balance between these two mechanisms may serve to control pseudopod formation and cell spreading and migration.

Pseudopod projection and adhesion are closely associated with expression of adhesion molecules. There is a possibility that cGMP indirectly enhances pseudopod retraction in response to shear stress via an effect on adhesion molecules. Among several adhesion molecules, firm adhesion of neutrophils during migration on substrates is mainly dependent on CD18. Therefore, we tested the CD18 expression and the influence of cGMP on the shear stress response. Although the CD18 expression on circulating neutrophils is downregulated by shear stress, cGMP has no effect on the expression after shear application (Figure 4B). Thus, the ability of cGMP to regulate the pseudopod retraction in response to shear stress may be independent of CD18 expression. Interestingly, in the absence of shear stress, 8-Br-cGMP has no effect on pseudopod formation of nonstimulated leukocytes, whereas the cGMP analog suppresses CD18 expression (Table 1, Figures 4A and 4B). In contrast, in the presence of shear stress, 8-Br-cGMP enhances the pseudopod retraction but not the downregulation of CD18 by fluid stress (Figures 4A and 4B). We demonstrated that fluid shear stress regulates both pseudopod formation and CD18 expression in leukocytes. Shear stress may play a role in inflammation via the regulation of CD18 expression as well as pseudopod retraction. Additional studies about changes in expression and localization of CD18 in adherent leukocytes in response to shear stress are needed.

One of the main mechanisms to increase intracellular cGMP levels in leukocytes is by NO. In addition to cGMP analogs, the NO donors SNAP and SIN-1 enhanced the neutrophil response to shear stress in the presence of PAF and FMLP (Figure 3). Although NO also acts as a scavenger of superoxide, the enhancement of the shear stress response by NO donors may be attributed to an increase in cGMP in neutrophils rather than the scavenging of superoxide, given that SOD by itself did not enhance the response to shear stress. The effect of NO donors on the fluid stress response is, however, mildly enhanced by SOD (Figure 3), probably due to the ability of SOD to scavenge superoxide derived from neutrophils and thereby increase NO levels.

Because NO is mainly produced by endothelial cells in the circulation, the endothelial cell may serve as a regulator for the shear stress response of leukocytes. To determine whether endothelium-derived NO regulates the shear stress response, we examined leukocytes of eNOS (2/2) mice, in which endothelial cells no longer produce NO synthesized by eNOS, with lower levels of NO in the blood (Table 2). Compared with leukocytes in wild-type mice, the cells in eNOS (2/2) mice exhibited a reduced response to shear stress (Figure 4C). The evidence suggests that endothelium-derived NO enhances the shear stress response of leukocytes in the circulation.

To clarify mechanisms of the shear stress response controlled by cGMP, NO, and inflammatory stimulators in vivo, we also examined pseudopod formation by leukocytes in rat mesentery venules after reduction and restoration of physiological shear stresses. Nonstimulated leukocytes project pseudopods during vessel obstruction but retracted them immediately after restoration of fluid stress. After retraction of the pseudopods, the leukocytes were washed away from the endothelium by the bloodstream (Figures 5A through 5C). Pseudopod retraction in response to fluid stress could be suppressed by application of
PAF. NOS inhibition by L-MNA strongly enhanced the ability of PAF to attenuate the shear stress response of leukocytes (Figures 5D through 5F). In the presence of an inflammatory stimulus, leukocytes may need higher levels of cGMP to maintain the shear stress response than under normal conditions. Inhibition of NOS, therefore, may cause an imbalance between cGMP and inflammatory stimulators in controlling pseudopod formation under normal shear stresses. This hypothesis is further supported by experiments that show that 8-Br-cGMP not only diminished the ability of PAF to suppress the shear stress response in vivo but also reversed the ability of L-MNA to block the fluid shear stress response. Because L-arginine also reversed the blockade of the shear stress reaction by L-MNA, L-MNA may influence the shear stress response by inhibition of NO production. The release of NO can be enhanced by the induction of NOS in neutrophils.14 Yet, the shear stress response may require mainly endothelium-derived NO, because in the absence of endothelial cells, NOS inhibitors (L-MNA and L-NNAME) did not affect the shear stress response in vitro both with and without inflammatory stimulators (Figure 4B). In eNOS (−/−) mice, the shear stress response of leukocytes was reduced even in the absence of the endothelium in vitro (Figure 4C), because eNOS (−/−) mice may have lower levels of cGMP in leukocytes due to lower NO production in the circulation.

These in vitro and in vivo data suggest that NO may regulate the leukocyte-pseudopod formation in response to shear stress in the circulation via cGMP. The enhancement of the shear stress response by NO is likely to suppress pseudopod projection and spreading of leukocytes on microvascular endothelium and consequently the level of the inflammatory reaction. Therefore, the regulation of the shear stress response by NO may be one of the key factors to modulate leukocyte-endothelial cell interactions, although NO may have multiple additional effects on leukocyte-endothelial cell interactions.13,15,16 The effect of NO on pseudopod formation and spreading in the absence of flow is still uncertain. NO was reported to induce monocyte and granulocyte rounding on a substrate via a morphine receptor.17 NO reduced F-actin formation in neutrophils,18,19 whereas cGMP increases it.19 One report showed that NO had no direct effect on PAF-dependent neutrophil adhesion in vitro in the absence of shear stress,20 whereas others reported that NO attenuated neutrophil and monocyte adhesion.18,19,21 In the present study, we would like to emphasize the effect of cGMP in the presence of blood flow because the presence of fluid shear stress is the normal condition for peripheral leukocytes, and inflammation frequently occurs under conditions of blood flow, unless vessels are occluded. Endothelial cells that produce NO may raise cGMP in attached leukocytes and maintain their shear stress response by suppression of pseudopod projection. In contrast, endothelial cells that produce lower levels of NO are more likely to permit spreading and attachment of leukocytes in the presence of normal fluid stresses. It is of interest to note that endothelial cells increase NO production in response to shear stress,1 leading in turn to an enhancement of the shear stress response of leukocytes. These observations may be important for the understanding of attachment and spreading of leukocytes on vascular endothelium, lymphocyte recirculation, inflammation, and immunoprotection.
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Circ Res. 2000;86:e13-e18
doi: 10.1161/01.RES.86.1.e13

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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