G protein-coupled receptors serve as mechanosensors for fluid shear stress in neutrophils

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THE INFLAMMATORY CASCADE is the basis for tissue repair and wound healing. Increasing evidence, however, shows that subclinical chronic inflammation is an important pathogenic mechanism leading to the development of cardiovascular disease. One of the key events in inflammation is neutrophil activation with pseudopod projection, oxygen free radical production, expression of membrane adhesion molecules, and degranulation. Pseudopod projection per se causes significant flow reduction in the microcirculation and stasis in capillaries, whereas retraction of pseudopods reduces microvascular entrapment and inflammation (16, 34, 38). The state of activation of circulating leukocytes can be controlled by a number of mechanisms traditionally thought of mostly in terms of inflammatory mediators. We demonstrated that fluid shear stress without inflammatory mediators also regulates leukocyte activation, such as pseudopod formation and microcirculatory blood flow (29). In the static state without fluid shear stress, neutrophils have the ability to form pseudopods after contact with different types of surfaces, including coverslips or vessel walls, and they are constitutively activated. Fluid shear controls spontaneous activity of the Rho family small GTPase, Rac, during pseudopod retraction. Deactivation of Rac by fluid shear plays a critical role in leukocyte circulation without entrapment in the capillary network (28). However, the sensor elements in the cell membrane that link extracellular fluid shear stress to intracellular signaling cascades are currently unknown.

Fluid shear controls the morphology of neutrophils differently from, for example, endothelial cells that elongate in the direction of fluid shear or smooth muscle cells that elongate perpendicular to shear direction (8, 22, 40). The specificity of the response to fluid shear among different cell types suggests that specific mediators on the cell membrane are involved in fluid shear sensing and transduction mechanisms. Thus we hypothesized that G protein-coupled receptors (GPCRs) serve as mechanosensors that respond to fluid shear stress in neutrophils. GPCRs in the plasma membrane exhibit constitutive activity, which is the ability to assume an active conformation in the absence of agonist, sufficient to promote intracellular stimulation of downstream effectors (36). GPCR constitutive activity in the leukocyte plays a critical role in its pseudopod formation. Neutrophils express several Gi protein-coupled chemoattractant receptors, including formyl peptide receptor (FPR), and activated receptors play an important role in cell migration via Rho family small GTPases (1, 32, 41). Because FPR exhibits relatively high constitutive activity among other chemoattractant receptors and is expressed predominantly in neutrophils and monococytes but less in other cell types (21), FPR may be a mechanosensor candidate for leukocytes.

In this study, we have used human promyeloid HL60 cells differentiated by DMSO into neutrophils to facilitate the use of transfection methods and allow the expression of exogenous proteins (28, 33). HL60 cells exhibit the ability to differentiate into morphologically mature myeloid cells with many of the markers and capabilities of neutrophils (9, 17, 33). Differentiated HL60 cells have been used extensively as a model system for studying neutrophil function, and they exhibit a robust fluid shear response (28).

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We report herein the role of GPCRs as mechanosensors in fluid shear stress-induced pseudopod retraction in neutrophils. Fluid shear may control pseudopod formation via a decrease in GPCR constitutive activity and thereby maintain stable circulation of leukocytes.

MATERIALS AND METHODS

Biological materials and reagents. Human promyelocytic leukemia HL60 cells [American Type Culture Collection (ATCC), Manassas, VA] were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS (Mediatech, Herndon, VA) in 5% CO2 at 37°C. HL60 cell differentiation was induced by treatment with 1.4% DMSO (ATCC) for 5 days. The mouse anti-FPR MAb was obtained from BD Pharmingen (San Diego, CA). HBSS and N-formyl-NeuL-Leu-Neu-Neu-Tyr-lys fluorescent derivative were obtained from Invitrogen. The protease inhibitors, PMSF, aprotinin, and leupeptin, the chemoattractant, formyl-methionyl-leucyl-phenylalanine (fMLP), pertussis toxin (PTX), and monensin were purchased from Sigma Chemical (St. Louis, MO).

Flow chamber application in a flow chamber. Flow chamber studies were performed as previously described (28). Cells were suspended in RPMI 1640 containing 10% FBS and loaded into the flow chamber. Laminar shear stress (5 dyn/cm²) was generated by perfusion of HBSS (pH 7.4 adjusted with 10 mM HEPES containing Ca²⁺ and Mg²⁺ at 1 mM each) with an automated syringe pump (Harvard Apparatus, Holliston, MA) attached to the inlet side of the flow chamber. The cell responses that we observed in the present study were all evoked by physiological but comparatively small fluid shear stress levels (5 dyn/cm² or ∼5/981 cmH2O) that were far below the values required to achieve a passive viscoelastic response (∼100–1000 dyn/cm²) (42). Images of the cells were recorded using a ×60 magnification lens objective and a 1.9 numerical aperture on a charge-coupled device camera (model VI-470; Optronics, Goleta, CA) and stored digitally (Scion Image software; Scion, Frederick, MD). Images were recorded during 10 min of fluid shear stress and for 10 min during the recovery phase. While neutrophils adhere to a coverslip, they project cytoplasmic regions designated as pseudopods, regardless of their particular shapes, such as veil-like lamellipodia, fingerlike filopodia, and uropodia at the tailing edge. Each cell contour was outlined manually, and the area within the outlined shape was computed (Scion Image software). This measurement is referred to as projected cell area.

Transient transfection of Gi proteins. Gi protein construction was prepared as previously described (5). Enhanced yellow fluorescent protein (YFP) (F46L) was inserted into the A-B loop within the α-helical domain of the Giα1 subunit. Gβγ2 subunits were fused to cyan fluorescent protein (CFP) on the NH₂ terminus of Gβ1 (CFP-N-Gβ1). A heterotrimERIC formation of Giα1-CFP, CFP-N-Gβ1, and Gγ2 can cause an increase in the fluorescence resonance energy transfer (FRET)-to-CFP ratio. HL60 cells were cultured in the presence of 1 M fMLP.

Fluorescence imaging. For FRET imaging, we used an inverted microscope equipped with a ×60 magnification oil-immersion lens objective, a cooled charge-coupled device camera, a filter changer, a 440DF20 excitation filter, a 455DFLP dichroic mirror, and two emission filters (535DF25 and 480DF30). Illumination time was set to 300–400 ms, and images were captured once per minute during the experiment and digitally analyzed (Simple PCI software, version 5.2; CompiX Imaging System, Cranberry Township, PA). The FRET signal was determined as the ratio of the YFP-to-CFP (FRET-to-CFP) emission. A background image was recorded under exactly the same conditions without cells, and the background fluorescence intensity was subtracted.

Stable transfection of HL60 cells. FPR small interfering RNA (siRNA) or random RNA sequence (mock) were constructed into the pSHRN-RetroQ retroviral vector (Clontech Laboratories, Mountain View, CA) (20). HL60 cells were transfected using electroporation (gene pulsar; Bio-Rad Laboratories, Hercules, CA). Cells (2 × 10⁶) were centrifuged and resuspended in 400 μL of Opti-MEM reduced serum medium (Invitrogen). Vector (10 μg) was added to the cells and preincubated for 5 min on ice. The cells were then subjected to a single 200-V pulse from a 960-μF capacitor and returned to 10 mL of culture medium. The next day, 1 μg/mL puromycin was added to the medium. As the selection proceeded, the cells were centrifuged and resuspended in fresh medium (containing puromycin) at 3- to 5-day intervals. As the cell density began to increase between 13 and 16 wk posttransfection, the medium was changed as needed to maintain cell density at <2 × 10⁶ cells/mL. FPR cDNA was transfected as described by Prossnitz et al. (33).

Ligand binding assay with flow cytometry. The cells were harvested by centrifugation and resuspended in cold HBSS (10⁶ cells/mL). Binding was conducted with N-formyl-NeuL-Leu-Neu-Neu-Tyr-lys fluorescent derivative at 10 nM concentration. After incubation for 20 min on ice, ligand binding was analyzed using flow cytometry (Becton Dickinson, San Jose, CA). Debris and dead cells were excluded by gating forward and side scatter. Nonspecific binding was determined in the presence of 1 M MPMPI.

RNA isolation and RT-PCR. RT-PCR was performed as described previously by Le et al. (20). DNA-free total RNA was extracted from cells (RNeasy Mini kit; Qiagen, Valencia, CA). Total RNA (0.1 μg) was reverse transcribed (iScript cDNA synthesis kit; Bio-Rad Laboratories). PCR (GeneAmp PCR system; PerkinElmer, Boston, MA) was performed using AccuPrime Taq DNA Polymerase (Invitrogen). The sense and antisense primers were specially designed from the coding regions of FPR genes (20). β-Actin was used as a control. PCR products were visualized using ethidium bromide staining in 1.2% agarose gel.

Statistical analysis. Data are presented as means ± SE. For comparison of groups, two-way repeated-measures ANOVA was performed and the Bonferroni-Dunn test was used for post hoc analysis. Comparisons between groups of maximum response (%) were conducted using an unpaired Student’s t-test as summarized in Table 1.

RESULTS

Fluid shear decreases Gi protein constitutive activity in differentiated HL60 cells. The FRET technique was used to visualize Gi protein activity. Giα1-CFP, CFP-N-Gβ1, and Gγ2 were cotransfected transiently in the differentiated HL60 cells, and the ratio of FRET to CFP was measured during the application of fluid shear stress. With this construction, the binding of the Gα subunit to the Gβγ subunit causes a FRET signal (5). As a control experiment for differentiated HL60 cells transfected with Gi1 proteins, FPR was stimulated using
interfering RNA-transfected differentiated HL-60 cells.

transfected differentiated HL-60 cells; D-siRNA, formyl peptide receptor small formyl peptide receptor-transfected differentiated HL60 cells; D-Mock, mock-formyl peptide receptor-transfected undifferentiated HL60 cells; DHLGFP, green fluorescent protein-transfected undifferentiated HL60 cells; HLFPR, pertussis toxin; HL, HL60 cells; DHL, differentiated HL60 cells; HLGFP, pertussis toxin.

Values are means ± SE. *P < 0.05 in 2-way ANOVA column indicates significant difference of maximum response between control and treatment groups using 2-way repeated-measures ANOVA. *P < 0.05 in Maximum Response column indicates significant difference of %maximum response between control and treatment groups using an unpaired Student’s t-test. PTX, pertussis toxin; HL, HL60 cells; DHL, differentiated HL60 cells; HLGFP, green fluorescent protein-transfected undifferentiated HL60 cells; HLFPR, formyl peptide receptor-transfected undifferentiated HL60 cells; DHLGFP, green fluorescent protein-transfected differentiated HL60 cells; DHLFPR, formyl peptide receptor-transfected differentiated HL60 cells; D-Mock, mock-transfected differentiated HL60 cells; D-siRNA, formyl peptide receptor small interfering RNA-transfected differentiated HL-60 cells.

Table 1. Summary of cell response to fluid shear

<table>
<thead>
<tr>
<th>Group</th>
<th>2-Way ANOVA</th>
<th>Maximum Response, %</th>
<th>p Value</th>
<th>Figure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control</td>
<td>PTX</td>
<td>&lt;0.05</td>
<td>2A</td>
</tr>
<tr>
<td>Ethanol vs. monensin</td>
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<td>Monensin</td>
<td>&lt;0.05</td>
<td>2B</td>
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<tr>
<td>HL vs. DHL</td>
<td>HL</td>
<td>DHL</td>
<td>&lt;0.05</td>
<td>3C</td>
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<tr>
<td>HLGFP vs. HLFPR</td>
<td>HLGFP</td>
<td>HLFPR</td>
<td>&lt;0.05</td>
<td>4C</td>
</tr>
<tr>
<td>DHLGFP vs. DHLFPR</td>
<td>DHLGFP</td>
<td>DHLFPR</td>
<td>&lt;0.05</td>
<td>4D</td>
</tr>
<tr>
<td>D-Mock vs. D-siRNA</td>
<td>D-Mock</td>
<td>D-siRNA</td>
<td>&lt;0.05</td>
<td>5C</td>
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Fluid shear-induced reduction of cell spreading is associated with deactivation of GPCR constitutive activity. Fluid shear caused rapid and significant rounding of the differentiated HL60 cells as detected by reduction of the projected cell area (28). To examine the effects of inverse agonist of GPCR constitutive activity on fluid shear-induced cell response, differentiated HL60 cells were treated overnight with 20 ng/ml PTX in culture medium and then subjected to fluid shear stress in the flow chamber. In the presence of PTX, the cells not only responded less to fluid shear but also demonstrated a marked increase in the degree of cell spreading during the last 4 min of shear stress exposure (Fig. 2A). The rise in cytosolic Na+ concentration ([Na+]cyt) by pretreatment with monensin, a Na+ ionophore, served to block the fluid shear stress-induced reduction of cell spreading (Fig. 2B), suggesting that fluid shear attenuates cell spreading via control of GPCR constitutive activity.

Response to fluid shear in undifferentiated HL60 cells. Undifferentiated HL60 cells do not express chemoattractant receptors in the plasma membrane, including the FPR (33). After differentiation, HL60 cells strongly express FPR mRNA and FPR in the plasma membrane (Fig. 3, A and B). Before application of fluid shear stress, differentiated HL60 cells had been migrating on the coverslip and spreading their cytoplasm, and fluid shear stress induced a rapid and significant reduction of projected cell area. In contrast, undifferentiated HL60 cells had a low ability to form pseudopods while adhering to the wall of the flow chamber and demonstrated no detectable response to fluid shear stress (Fig. 3C).

FPR transfection in HL60 cells induces pseudopod formation and restores the cell response to fluid shear. To examine whether fluid shear stress causes cell retraction via FPR, we

![Fig. 1. Fluid shear decreases Gi protein constitutive activity in differentiated HL60 cells.](http://ajpcell.physiology.org/)

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transfected the FPR cDNA in undifferentiated HL60 cells using electroporation (33). cDNA encoding only green fluorescent protein (GFP) was transfected into the cells as a control. FPR cDNA transfection enhanced the expression of FPR protein on the surface membrane in undifferentiated HL60 cells, which, without FPR cDNA transfection, showed low levels of FPR. The expression levels starting from the lowest levels were: undifferentiated HL60 cells < differentiated HL60 cells < FPR-transfected undifferentiated HL60 cells < FPR-transfected differentiated HL60 cells (Fig. 4A).

Importantly, expression of FPR in undifferentiated HL60 cells causes pseudopod projection (Fig. 4B) and pseudopod retraction during fluid shear (Fig. 4C). Furthermore, overexpression of FPR by differentiation of FPR-transfected HL60 led to even stronger pseudopod retraction during fluid shear compared with differentiated HL60 cells transfected only with GFP (Fig. 4D).

Inhibition of FPR expression attenuates fluid shear-induced response. To investigate whether GPCRs other than FPR might facilitate fluid shear-induced reduction of cell spreading, we examined whether FPR siRNA could affect the cellular response to fluid shear. Undifferentiated HL60 cells were stably transfected with FPR siRNA by electroporation. Mock-transfected cells were used as the control. Transfected cells were differentiated and then used for the flow chamber study. FPR siRNA transfection in HL60 cells successfully downregulated the levels of FPR mRNA (Fig. 5A) and surface membrane FPR expression (Fig. 5B) compared with mock-transfected cells. FPR siRNA-transfected differentiated HL60 cells, which expressed other receptors except for FPR, significantly decreased the cell response to fluid shear compared with mock-transfected differentiated HL60 cells (Fig. 5C).

These results suggest that FPR plays a central role in pseudopod retraction during fluid shear.

DISCUSSION

Altogether, these results indicate that in neutrophils, the fluid shear stress-induced signaling pathway with reduction of cell spreading due to pseudopod formation is initiated by deactivation of the constitutive activity of GPCR. FRET permits the detection of protein-protein interactions in living cells and also enables determination of G protein activity (5). FPR has the ability to couple to all subtypes of Gi1/Gi2/Gi3 proteins, including Gi1 (36, 39), although it couples mainly to Gi2 and Gi3 in HL60 cells (14, 31). In this study, we tested Gi1 protein activity because Gi1 generates much higher FRET signals compared with background, whereas Gi2 and Gi3 do not generate a sufficient FRET signal-to-noise ratio (12). We also observed a significant increase in the FRET ratio after stimulation with fMLP, an agonist for FPR (Fig. 1A). Fluid shear stress sig-
icantly decreased the FRET ratio within seconds in differentiated HL60 cells (Fig. 1B), indicating a reduction in GPCR constitutive activity. In this study, we used differentiated HL60 cells instead of FPR siRNA-transfected differentiated HL60 cells, because we observed enhanced cell apoptosis after cotransfection of $G_i$ proteins into the FPR siRNA-transfected HL60 cells.

The two-state model assumes that GPCRs exist between an inactive state (R) state and an active state (R*) (19, 24, 25, 36). The R-to-R* isomerization of GPCRs during constitutive activity can occur spontaneously, i.e., independently of agonist. Basal constitutive activity of GPCRs can be blocked by inverse agonists but not by receptor antagonists. PTX serves to inhibit constitutive activity of $G_i$ protein-coupled receptors (7, 18, 23), and the monovalent cation $Na^+$ acts as an allosteric inverse agonist that stabilizes the R state and diminishes basal G protein activity (6, 10, 15, 36). Fluid shear stress rapidly reduced cytoplasmic spreading of differentiated HL60 cells. In the presence of PTX, the pseudopod retraction during fluid shear was minimized and cell spreading was observed (Fig. 2A). The reason for this phenomenon may be that the blockade of $G_{i_{0_{0}}}$ by PTX increased the activities of other types of $G$ proteins (2, 4) and subsequently caused pseudopod formation via fluid shear stress. The rise in cytosolic $[Na^+]_{cyt}$ upon monensin treatment diminished the fluid shear-induced reduction in cell spreading (Fig. 2B). These results suggest that in differentiated HL60 cells, fluid shear decreases GPCR constitutive activity together with pseudopod retraction.

Differentiated HL60 cells expressed FPR in the plasma membrane (Fig. 3, A and B), migrated on a coverslip, and spread their cytoplasm before shear stress was initiated. Fluid shear stress induced a rapid and significant reduction of projected cell area (Fig. 3C). This fluid shear stress response is largely missing in undifferentiated HL60 cells because of the absence of surface membrane receptors (e.g., FPR) required to initiate pseudopod projection (33). Expression of only FPR in undifferentiated HL60 cells restored their ability to project pseudopods that they retract under the influence of fluid shear.
In contrast, suppression of only FPR expression in differentiated HL60 cells attenuated their ability to respond to fluid shear stress (Fig. 5). These results thus suggest that the FPR serves as a mechanosensor for fluid shear.

In addition to FPR, two other receptor types in the FPR family have been identified: FPR-like 1 (FPRL1) and FPR-like 2 (FPRL2). FPR and FPRL1, but not FPRL2, are detected in neutrophils. FPR is defined as a high-affinity fMLP receptor (nM range); FPRL1 is characterized as a low-affinity fMLP receptor (μM range); and FPRL2 does not bind or respond to fMLP (21). In this study, we focused on FPR because its constitutive activity has been well characterized (36, 37). Neutrophils have other chemoattractant receptors in the plasma membrane, including complement components C5a and C3a receptors, platelet-activating factor receptor, and leukotriene B4 receptor. These receptors exhibit constitutive activity (36, 37). FPR-transfected undifferentiated HL60 cells expressed more FPR in the surface membrane than differentiated HL60 cells did (Fig. 4A), but the maximum response induced by fluid shear stress in FPR-transfected undifferentiated HL60 cells (Fig. 4C) was not as strong as that in differentiated HL60 cells (Fig. 3C). Therefore, other receptors may also mediate cell retraction in response to fluid shear stress.

Fluid shear may control FPR constitutive activity by 1) fluid shear induced influx of Na+ and 2) a direct change in the conformation of FPR. A number of other sensory molecules for fluid shear were proposed mainly for vascular endothelium, such as heparan sulfate proteoglycans (11, 30), caveolae (35), fluid shear were proposed mainly for vascular endothelium, conformation of FPR. A number of other sensory molecules for fluid shear. Spontaneous association between opioid receptors and GTP-binding regulatory proteins in native membranes: specific regulation by antagonists and sodium ions. Mol Pharmacl 37: 383–394, 1990.

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