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Objective—Atherosclerosis-prone regions of arteries are characterized by complex flow patterns where the magnitude of shear stress is low and direction rapidly changes, termed disturbed flow. How endothelial cells sense flow direction and how it impacts inflammatory effects of disturbed flow are unknown. We therefore aimed to understand how endothelial cells respond to changes in flow direction.

Approach and Results—Using a recently developed flow system capable of changing flow direction to any angle, we show that responses of aligned endothelial cells are determined by flow direction relative to their morphological and cytoskeletal axis. Activation of the atheroprotective endothelial nitric oxide synthase pathway is maximal at 180° and undetectable at 90°, whereas activation of proinflammatory nuclear factor-κB is maximal at 90° and undetectable at 180°. Similar effects were observed in randomly oriented cells in naive monolayers subjected to onset of shear. Cells aligned on micropatterned substrates subjected to oscillatory flow were also examined. In this system, parallel flow preferentially activated endothelial nitric oxide synthase and production of nitric oxide, whereas perpendicular flow preferentially activated reactive oxygen production and nuclear factor-κB.

Conclusions—These data show that the angle between flow and the cell axis defined by their shape and cytoskeleton determines endothelial cell responses. The data also strongly suggest that the inability of cells to align in low and oscillatory flow is a key determinant of the resultant inflammatory activation. **(*Arterioscler Thromb Vasc Biol*. 2013;33:2130-2136.)**

Key Words: atherosclerosis ■ hemodynamics ■ mechanotransduction, cellular

Fluid shear stress from blood flow plays a key role in vascular physiology and pathology through its effects on vascular endothelial cell (EC) function.[1–5] Atherosclerotic lesions occur preferentially at regions of flow disturbance (ie, bifurcations, branch points, and regions of high curvature).[6,7] These regions are characterized by lower shear stress magnitude and complex changes in flow direction during the cardiac cycle. Bypass grafting and stenting can also introduce regions of disturbed flow, which correlate strongly with neointimal hyperplasia and atherosclerotic lesions[8–11]; indeed, there has been considerable effort to reduce flow disturbance from these interventions.[11–13] In vitro studies on ECs have demonstrated both proinflammatory effects of disturbed flow and suppressive effects of high steady or pulsatile laminar flow.[1,14] These results have led to the concept that flow patterns critically influence the initiation of atherosclerosis, in-stent restenosis, and bypass graft failure.[3–5]

Atherosclerosis-prone regions in vivo also strongly correlate with the failure of ECs to elongate and align.[15–17] Alignment has been proposed to be a mechanism by which cells adapt to flow and downregulate inflammatory pathways,[14,18] but there are few data to directly support this notion. Recently, it was shown that cell alignment itself decreases inflammatory signaling in ECs even in the absence of flow.[19] However, a causal connection to flow has not been established.

Many aspects of fluid shear stress, such as magnitude,[20,21] and temporal and spatial gradients[22–25] have been studied for their effects on vascular ECs, but the effects of flow direction are poorly understood. Most previous studies focused on the effects of flow reversal, attributable, in part, to the absence of in vitro systems that provide well-defined changes in flow direction other than 180°.[26] Flow reversal on shear stress–aligned ECs changed the expression levels of multiple growth and inflammatory genes, including platelet derived growth factor and nitric oxide synthase 3.[27] Nitric oxide (NO) is reduced in reverse flow compared with forward flow with same magnitude in ex vivo porcine arteries.[28] Flow reversal on prealigned cells affects microvascular permeability[29] and cell–cell junction inclination.[30]

However, realistic in vivo local shear stresses at regions of disturbed flow can be multidirectional because of complex flow patterns, such as time-varying vortices and helical flows.[31–37] For example, shear stress at the side walls of the proximal internal carotid artery changes direction sharply during systole over a range of 70°.[33] Formation of aneurysms after repair of aortic coarctation and the distal anastomotic intimal hyperplasia of vascular bypass grafts also correlate strongly with shear stresses

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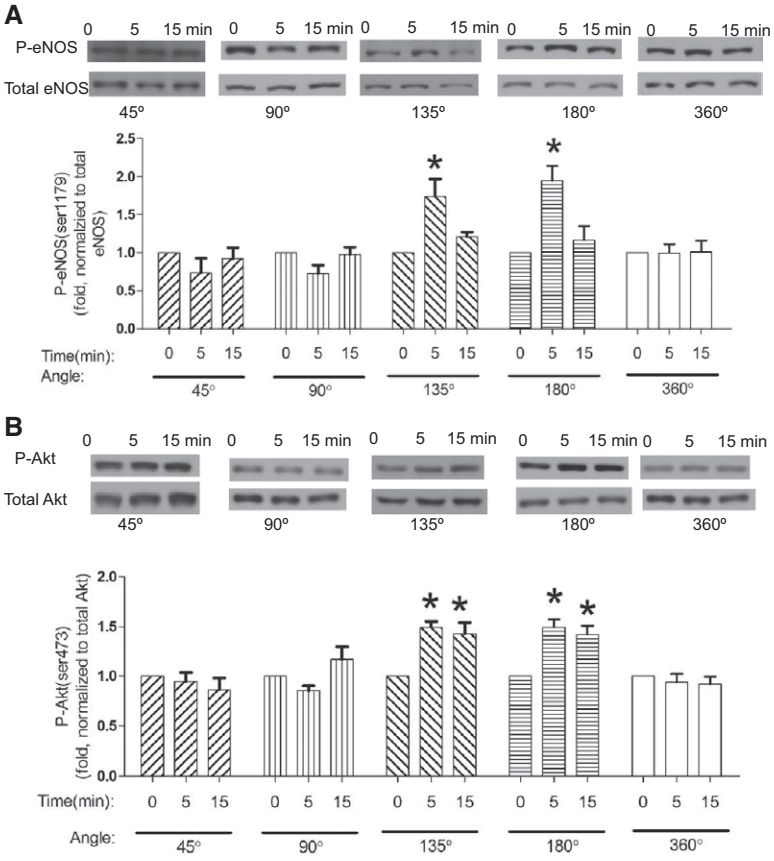

Figure 1. Activation of endothelial nitric oxide synthase (eNOS) and Akt after changing flow direction. Cells aligned in flow for 24 hours were subject to a change in direction by 45°, 90°, 135°, 180°, and 360°. At the indicated times, cells were harvested. Phospho-eNOS (ser1179; **A**) and phospho-Akt (ser473; **B**) were assayed by Western blotting. Values are means±SEM, n=4; *P<0.05.

with significant off-axis components.[31,34] Efforts to study the effects of off-axis flow (angles other than 0° and 180°) included computational analysis of the effects of perpendicular flow (90°) on subcellular stress distribution in aligned cells[38] and EC morphology.[39] The effects of biaxial oscillatory shear stress on cell morphology have also been studied.[40] However, effects of flow direction on signaling pathways have not been examined.

Most in vitro flow systems use oscillatory shear along 1 axis to model the more complex atherogenic flows found in vivo.[26,41] Some systems have modeled complex, time-varying in vivo flow magnitude along 1 axis.[42,43] We, therefore, developed and validated a novel flow system that can change the direction of shear by any angle.[26] In the present work, we used this system to address how ECs respond to changes in the direction of flow. When ECs were prealigned with flow and then subjected to a single change of flow direction, activation of nuclear factor (NF)-κB, endothelial NO synthase (eNOS), and Akt had distinct directional requirements. Aligning cells on micropatterned fibronectin or analysis of randomly oriented cells in a monolayer indicated that the angle between flow direction and cell axis, defined by cell shape and F-actin, dictates these flow responses. The data, therefore, identify a central role for cell alignment in response to shear stress.

Materials and Methods

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

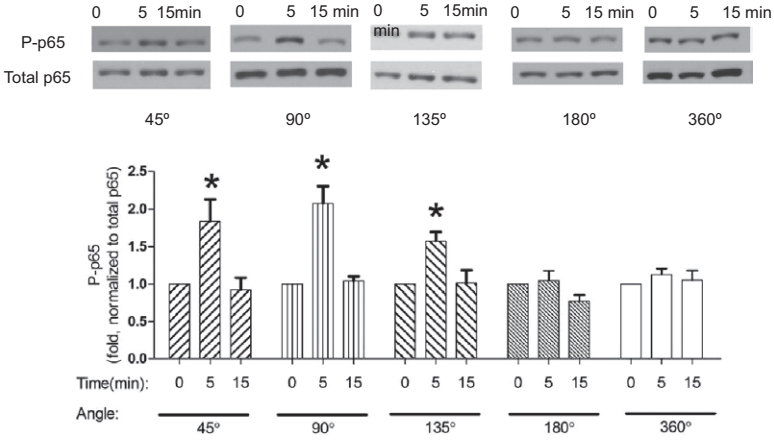

Figure 2. Activation of nuclear factor-κB after changing flow direction. Flow-aligned cells were subject to a change in flow direction as in Figure 1. Phospho-p65 was assayed by Western blotting. Values are means±SEM, n=4; *P<0.05.

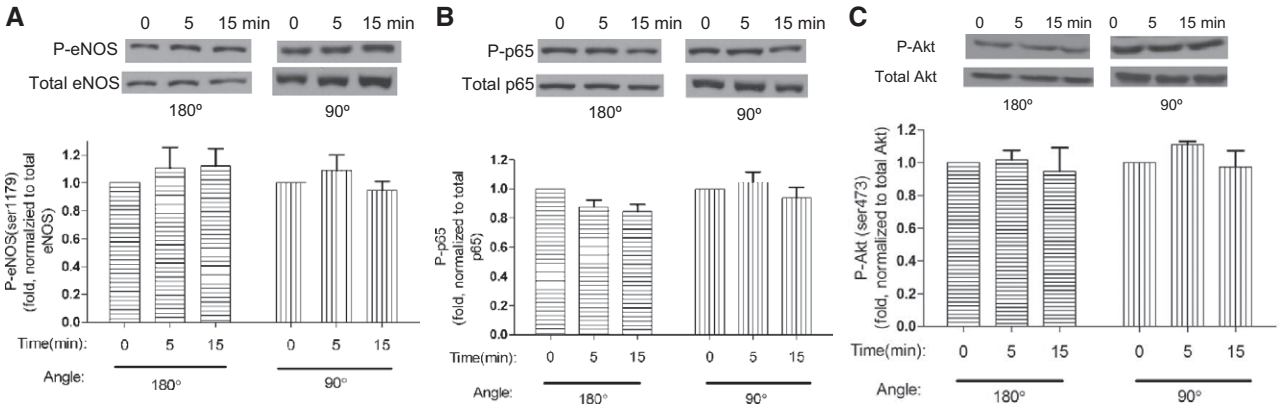

Figure 3. Responses of nonaligned cells to change of flow direction. Cells under flow for 2 hours were subject to a change in flow angle as in Figure 1. **A**, Phospho-endothelial nitric oxide synthase (eNOS). **B**, Phospho-p65. **C**, Phospho-Akt. Values are means±SEM, n=3.

Results

Responses of Flow-Aligned ECs to Flow Direction

Disturbed flow often involves complex changes in the direction of flow. To investigate how these might affect EC function, we subjected aligned bovine aortic endothelial cells to a single change in flow direction. We examined 4 critical pathways: NF-κB (p65), which is implicated in inflammation; eNOS, which is implicated in flow-dependent vasodilation and suppression of inflammation[44]; and Akt and AMP kinase, which have important roles in shear stress–induced cellular responses.[45,46] After alignment of bovine aortic endothelial cells under 24 hours of laminar flow, flow direction was changed to angles between 45° and 180°. A 360° rotation served as a control for the effects of rotation itself. We found that phosphorylation of eNOS and Akt was induced only by a change in shear to high angles and was maximal at 180º, whereas a 45° or 90° change in flow direction had no effect (Figure 1). Similar results for eNOS and Akt are consistent with the known role of Akt upstream of eNOS S1177.[47] In contrast, NF-κB activation, assessed by phosphorylation of p65 on Ser 536, was activated by lower angles, being maximal at 90° but unchanged after flow reversal (ie, 180°; Figure 2). A 360° rotation had no effect on any of these pathways, indicating that brief rotation itself did not stimulate these pathways. AMP kinase activation was negligible under all these conditions (data not shown).

Short-Term Shear Without Alignment

To determine whether these anisotropic responses require cell alignment, bovine aortic endothelial cells were preconditioned under flow for only 2 hours. At this time, NF-κB, eNOS, and Akt activity had decreased to levels well below their peaks at 30 to 60 minutes, but cells were not yet substantially aligned. Under these conditions, changing flow direction did not elicit significant changes in eNOS, p65, or Akt phosphorylation (Figure 3). These results show that cell alignment is a prerequisite for the anisotropic responses to changes in flow direction.

Responses of Elongated Cells in Naive Cell Monolayers to Flow

We next asked whether the polarity that determines responses to flow direction was because of the axis related to cell shape and the cytoskeleton or by another type of polarity induced by flow. In naive endothelial monolayers, a fraction of the cells are elongated, although randomly oriented. We therefore applied onset of flow (12 dynes/cm^2) to naive monolayers for 30 minutes and then assessed NF-κB activation by staining cells for p65. Activation was assessed by scoring nuclear translocation as a function of cell orientation relative to the flow direction. Cell orientation was defined as parallel (0°–30°), intermediate (30°–60°), or perpendicular (60°–90°) to the direction of flow. Cells that were perpendicular to the flow direction showed substantially higher nuclear p65 than intermediate or parallel (Figure 4B). Thus, in cells not

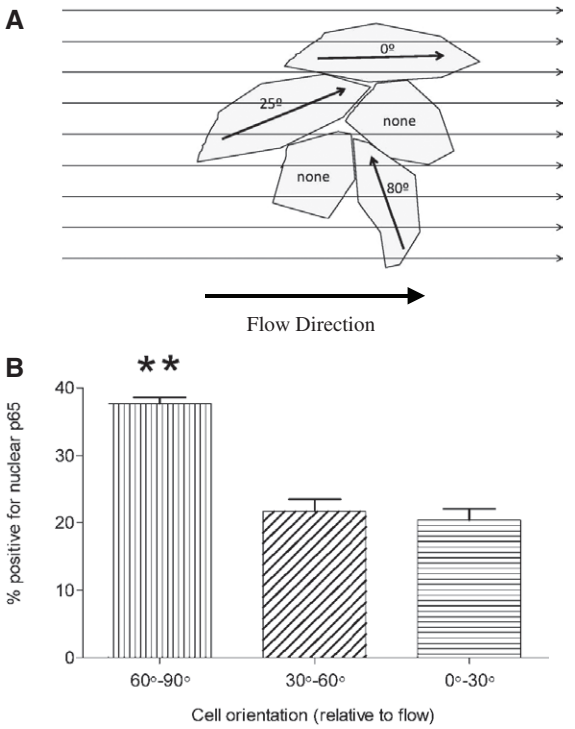

Figure 4. Responses of naive cells to the onset of flow. **A**, Cartoon of cells in a naive cell monolayer with different angles relative to the flow direction. **B**, Nuclear factor-κB nuclear translocation in elongated cells that were perpendicular (60°–90°), intermediate (30°–60°), or parallel (0°–30°) to the flow direction. Shear stress of 15 dynes/cm^2 was applied for 30 minutes. Values are means±SEM, n=3; $^{**}P<0.001$.

previously exposed to flow, the angle of the flow relative to the axis defined by cell shape is critical for NF-κB activation in individual elongated cells.

Responses of Micropattern-Aligned ECs to Flow Direction

Oscillatory flow, which is commonly used to model in vivo disturbed flow, does not induce cell alignment and induces sustained activation of NF-κB.[48] If cellular responses to oscillatory shear depend on the angle between flow direction and the cell axis in the same manner as for changes in flow direction or onset of flow, failure to align might be causal for NF-κB activation in oscillatory flow. To test this hypothesis, bovine aortic endothelial cells were plated on coverslips with micropatterned fibronectin lines, which induce cell alignment in the direction of lines without flow. Cells were then subjected to oscillatory flow (0.5±3 dynes/cm^2 for 2 hours) parallel or perpendicular to the fibronectin lines. NF-κB was activated to a higher extent in cells that were perpendicular to the flow direction than parallel (Figure 5). Activation of eNOS showed the opposite behavior, with higher activation by parallel compared with perpendicular flow (Figure 5B). Activation of Akt showed a similar trend as eNOS but did not reach statistical significance (Figure 5B). As before, AMP kinase activity was unchanged (data not shown).

The balance between NO and reactive oxygen species is a major determinant of endothelial function versus dysfunction and atherosclerotic progression in arteries.[49,50] We therefore measured their levels in cells on the micropatterned substrates. Perpendicular oscillatory shear stress induced higher reactive oxygen species and lower NO compared with parallel flow (Figure 6). These data strongly implicate effects of flow direction on the pathways that play major roles in determining endothelial function and disease.

Discussion

In vivo shear stress at regions of disturbed flow are often multidirectional because of complex patterns, such as time-varying vortices and helical flows.[31–37,51] Although the effects of shear stress on ECs have been extensively investigated in vitro, effects of flow direction are understudied as a result of the limitations of commonly used in vitro systems. We therefore used a recently developed flow system that enables changes in flow direction. Our results showed that eNOS and Akt were maximally activated by a 180° change in flow, essentially a single oscillation along the cell axis, whereas at this angle NF-κB activation was unchanged. In contrast,

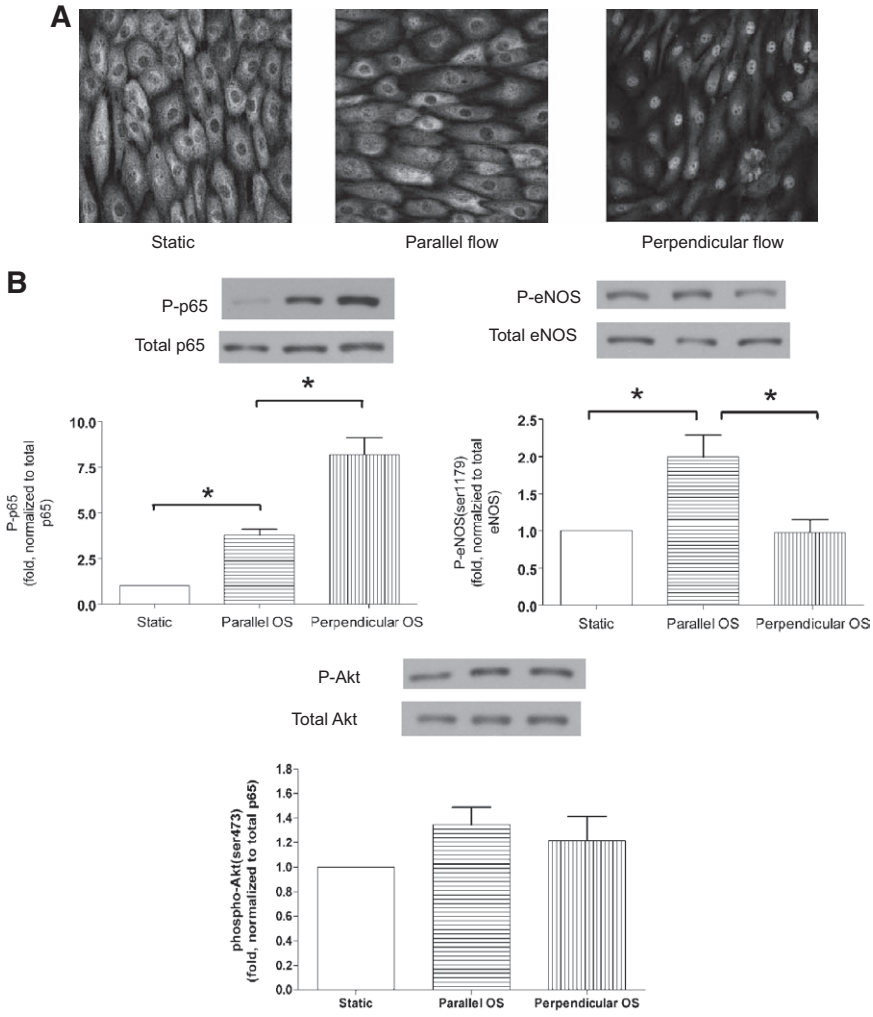

Figure 5. Response of micropattern-aligned cells to oscillatory flow. Cells aligned on micropatterned fibronectin lines were analyzed by staining for p65 and visualizing nuclear translocation. **A**, Images of cells without flow (**left**) and with 2 hours of oscillatory flow parallel (**center**) or perpendicular (**right**) to the lines. **B**, Cells were extracted and analyzed by Western blotting for phospho-p65 (**left**), phospho-endothelial nitric oxide synthase (eNOS; **right**), and phospho-Akt (**bottom**). Values are means±SEM, n=3; **P*<0.05. OS indicates oscillatory shear.

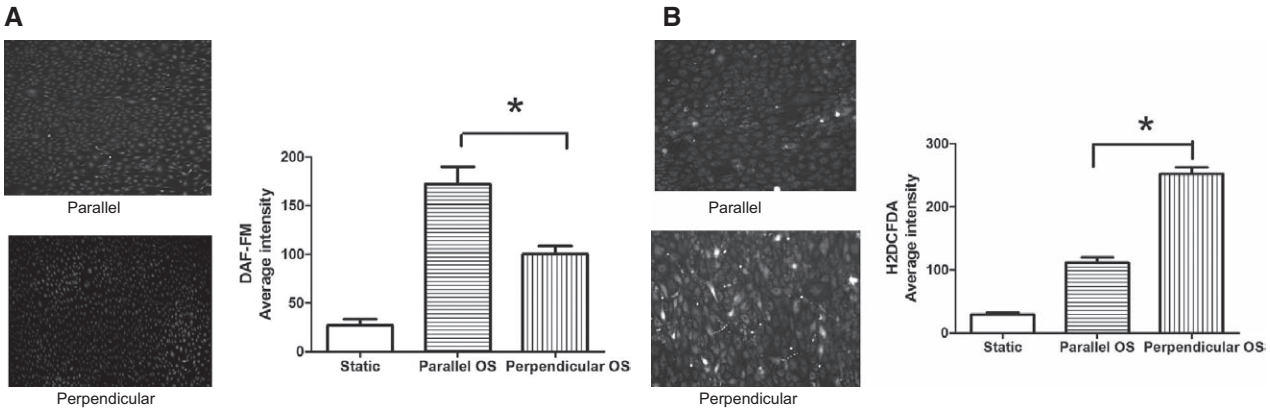

Figure 6. Reactive oxygen and nitric oxide production. Cells on micropatterned surfaces were subject to oscillatory flow for 1 hour. **A**, Diacetate (4-amino-5-methylamino- 2′,7′-difluorescein) detection of nitric oxide. **B**, Dichlorodihydrofluorescein diacetate (H2DCFDA) detection of reactive oxygen species. Images on the left and quantified values on the right are means±SEM, n=3; *P<0.05.

NF-κB was activated maximally by a 90° directional change in flow, which did not affect eNOS or Akt. These results led to the surprising conclusion that different flow angles stimulate activation of distinct pathways.

Passerini et al[27] reported that application of reverse flow of low magnitude decreased expression of eNOS after 6 hours; however, the differences in magnitude and time of analysis make it difficult to directly compare our results with theirs. Another study[28] found that reverse flow in porcine arteries stimulated NO production less well than forward flow because of superoxide production, which decreased NO availability.[28,52] However, to our knowledge, our data are the first to compare effects of forward, reverse, and perpendicular flow.

Further experiments with randomly aligned naive cells and with cells aligned on micropatterned substrates demonstrated that the cell axis that determines flow responses does not require flow but instead is determined by conventional cell shape. These results strongly suggest that cytoskeletal and adhesive structures provide the internal compass against which flow is measured. This idea is consistent with our previous study in which we observed that prealigned cells subject to a 90° rotation in flow remained aligned to each other but rotated toward the new flow direction at a constant speed.[26] This mechanism of realignment can be best explained by a model in which cells essentially compare flow direction with an internal axis and activate cytoskeletal and signaling pathways accordingly. Thus, several distinct cell responses support the concept that flow direction is sensed relative to the cytoskeletal axis of cells.

It is well established that oscillatory flow activates inflammatory pathways, such as NF-κB, that promote atherogenesis. Our results, however, showed that activation of NF-κB by oscillatory flow in micropatterned cells was also strongly dependent on flow direction. Interestingly, low laminar shear activates NF-κB nearly as well as oscillatory flow.[48] Low and oscillatory flow patterns are also distinct from high laminar shear in that they fail to induce alignment. These results suggest that an important reason why low and oscillatory flow are atherogenic is because cells fail to align in the flow direction so that many cells experience flow at high angles relative to their long axes. These effects govern whether flow stimulates activation of eNOS and production of NO versus production of reactive oxygen species and activation of NF-κB. The balance between these 2 pathways is an important determinant of vascular function, such that increased reactive oxygen species and decreased NO lead to endothelial dysfunction and promote atherosclerosis.[53,54] These direction-dependent differential responses of ECs to flow are, therefore, likely to be functionally important.

These results provide evidence that EC alignment under laminar flow is an important atheroprotective, adaptive process. They are also likely to be directly relevant to bypass surgery, where connecting arteries of different dimensions induce regions of disturbed flow downstream of the junction. It has been proposed that intimal hyperplasia in these regions is mainly caused by oscillatory flow.[55] Many studies[56] (mostly computational) have attempted to optimize the local shear stress to minimize intimal hyperplasia. However, none of these studies considered off-axis flow direction as a factor, which our data suggest is critical. Thus, our findings indicate that minimizing inflammatory responses and restenosis should require minimizing off-axis flow or enhancing cell alignment in the direction of flow.[57,58]

Future work will be directed toward identifying the molecular sensing mechanisms that mediate activation of distinct pathways depending on flow direction and in investigating the effects of complex, multidirectional flow patterns that more accurately model in vivo disturbed flow.

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Disclosures

None.

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Significance

Regions of arteries under low and disturbed fluid shear stress are susceptible to atherosclerosis, whereas regions under high laminar flow are atherosclerosis resistant. Susceptibility to atherosclerosis strongly correlates with poor endothelial alignment. In vitro, inflammatory flow profiles also fail to induce cell alignment. The current study demonstrates that signals stimulated by flow are determined by direction relative to the axis defined by cell shape and the cytoskeleton. Thus, flow parallel to the cell axis preferentially stimulates endothelial nitric oxide synthase activation and nitric oxide production, whereas perpendicular flow preferentially stimulates reactive oxygen and nuclear factor-κB activation. Thus, the same flow activates functionally opposite pathways depending on direction. These data fundamentally alter our understanding of how flow acts on the endothelium and provide a direct causal link between cell alignment and inflammatory activation. They also suggest new strategies for inhibiting restenosis in vascular grafts and other interventions.

MATERIALS AND METHODS:

Description of the flow system

A detailed description and validation of the flow system has been published previously [1]. Briefly, the device consists of a parallel plate flow chamber with a turntable mechanism built into the bottom plate. The chamber has a machine-milled polycarbonate top plate, a rectangular silicone gasket, and a polycarbonate bottom plate with a hole and a shaft fitted into the hole. A 40mm diameter circular glass coverslip onto which cells are seeded is held on top of the shaft by vacuum suction. The top plate, silicone gasket, and bottom plate are held together by screws. The glass coverslip is flush with the bottom plate surface to minimize flow disturbances. Cells were seeded only in the central 30 mm diameter area to avoid possible flow disturbances near the edges. To change the direction of fluid flow relative to the cell monolayer, the coverslip is rotated via the shaft. The flow chamber is connected to a peristaltic pump by two manifolds in the top plate through which medium enters and exits the channel. Culture medium circulates through the chamber and back to the reservoir in a closed loop. Flow pulsation from the pump is eliminated by a pulse dampener. Reynolds number for shear stress at 12 dynes/cm^2 is 91 (dynamic fluid viscosity for culture medium DMEM/F12 at 37°C is = 0.78 * 10^{-3} N.s/m^2). This flow system has been validated by computational and experimental approaches [1].

Cell culture and flow direction sensing by flow-aligned cells

Primary bovine aortic endothelial cells (BAECs) were purchased from VEC Technologies (Rensselaer, NY) and used from passage 8 to 10. Cells were maintained in DMEM/F12 media (Invitrogen 11320), supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals), 10 U/ml penicillin and 10 μg/mL streptomycin (Invitrogen). The central 30 mm of 40 mm diameter round glass coverslips were coated overnight with 20 μg/mL fibronectin using a silicone gasket to block the edges. Cells were seeded in this region and allowed to form a confluent monolayer overnight. The slides were then loaded onto the top of the shaft and immobilized by vacuum; the two plates and silicone gasket were held together by screws to form the parallel plate flow channel. Cells were aligned under laminar shear stress at 12 dynes/cm^2 for 24 hours. The shaft was then rotated by 45º, 90°, 135º, 180° or 360° and cells analyzed at 0, 5 and 15 minutes. (Signals returned to baseline by 30 min, thus, later times are not shown). To test whether results depend on cell alignment, a separate set of experiments were done for cell that were sheared for 2h and subsequently subjected to direction changes. To lower the baseline level of phosphorylation, cells were starved overnight in DMEM/F12 containing 0.5% FBS and sheared in the same medium.

Flow applied to naïve cell monolayers

BAECs seeded onto glass slides and allowed to form a confluent monolayer were starved overnight in DMEM/F12 containing 0.5% FBS, then subjected to flow at 12 dynes/cm^2 for 30 min (the peak time for NF-κB activation). Cells were then fixed and stained for p65. Geometrical parameters such as shape index and cell orientation were determined by manually tracing cell boundaries in ImageJ (NIH). Shape index is defined as $4\pi A/P^2$ (A: cell area, P: cell perimeter), thus, shape index is 1.0 for a circle and 0 for a line. An ellipse was fitted to the individual cell outlines and the angle of cell orientation (-90 degrees to + 90 degrees) was defined as the angle between the primary axis and the flow direction (flow direction is 0°; perpendicular direction is 90°). Cell that were round (shape index > 0.7) without a clear orientation were excluded from the

analysis. Cells were binned into 0°-30°; 30°-60°; 60°-90° and the percentage of cells with nuclear p65 was quantified. At least 300 cells from each coverslip were analyzed. Co-staining for both phospho-eNOS and total eNOS was technically difficult, thus, was not included in this system.

Oscillatory flow applied to micropattern-aligned cells

Patterns of parallel fibronectin lines (5um wide with 5um spacing) were stamped onto coverslips via soft lithography and microcontact printing [2]. Briefly, featured polydimethylsiloxane (PDMS) stamps were cast from a silicon master, inked with fibronectin, and placed in conformal contact with glass coverslips spuncoat with a thin layer of PDMS activated with UV ozone, and peeled off to leave protein patterned on the coverslip. Following microcontact printing, coverslips were incubated in pluronics F-127 to prevent protein adsorption and subsequent cell adhesion to non-printed areas. BAECs seeded on these patterns overnight formed a confluent monolayer aligned parallel to the fibronectin lines. Prior to experimentation, cells were starved for 1h in DMEM/F12 containing 2% FBS, which also served as the circulating medium for the shear experiments. Seeded coverslips were loaded into the flow chamber and subjected to parallel or perpendicular oscillatory shear stress at 0.5±3 dynes/cm^2 for 2h. (Examining longer times was problematic as cells detached from these substrates after 3-4h, presumably due to the reduced adhesive area on the patterned substrates).

Nitric oxide and reactive oxidative species assays

NO and ROS were measured using DAF-FM (Invitrogen #D-23844) and CM-H2DCFDA (Invitrogen #C6827), respectively. Briefly, micropattern-aligned BAECS were incubated with DAF-FM (5μM) or CM-H2DCFDA (5μM) in phenol red-free DMEM/F12 (Invitrogen 11039-021) at 37°C for 15 minutes, rinsed and left untreated or subjected to perpendicular or parallel oscillatory shear in serum-free and phenol red-free DMEM/F12 medium for 1h. Cells were then fixed in 2% paraformaldehyde for 5 min at 4ºC and imaged using a Zeiss LSM510 scanning confocal microscope with a 10x air objective. Average fluorescence intensity was determined. To determine baseline values, the eNOS inhibitor L-NAME (0.1mM) or the ROS scavenger catalase (300 U/mL) were added to cell culture medium after the incubation with DAF-FM for NO and ROS experiments, respectively. These values were used for background subtraction. At least 600 cells from each coverslip were included for each value.

Immunoblotting

Cells were lysed in Laemmli's buffer (0.1M Tris pH 6.8, 10% β-mercaptoethanol, 20% glycerol, 4% SDS). Samples were run on 8% SDS-PAGE gels and transferred to PVDF membranes (BioRad), which were blocked with 3% BSA in TBS containing 0.01% Tween-20. Blots were incubated with 1:1000 primary total-p65, phospho-p65, total-eNOS, phospho-eNOS(Ser1177) (Cell Signaling Technology) antibodies overnight at 4°C. Blots were washed, incubated with 1:5000 secondary goat anti-rabbit HRP antibody (Jackson Laboratories) for 1 hour and developed using ECL reagents (Pierce) and film (Kodak).

Immunofluorescence

For visualizing p65, cells were fixed with PBS containing 3.7% formaldehyde for 10 min and permeabilized with 0.2% Triton X-100 for 5 min. Coverslips were then incubated with 1:200

total p65 antibody (Cell Signaling, D14E12) overnight at 4C°, rinsed three times with PBS, incubated with Alexa Fluor 488 anti-rabbit secondary antibody for 1 hour at room temperature, rinsed three times with PBS, and mounted with Fluoromount G. Images were captured using a Zeiss LSM510 scanning confocal microscope with a 40x oil immersion lens.

Statistical analysis

At least three experiments were performed for each condition. Statistical differences between experimental groups were evaluated with analysis of variance (ANOVA) using Tukey post-hoc tests. *P*-values of 0.05 were considered statistically significant.

References:

1. Wang C, Lu H, Schwartz M. A novel in vitro flow system for changing flow direction on endothelial cells. *J Biomech.* 2012;45:1212-1218.

2. Tan J, Liu W, Nelson C, Raghavan S, Chen C. Simple approach to micropattern cells on common culture substrates by tuning substrate wettability. *Tissue Eng.* 2004;10:865-872.