Abstract
Endothelial cell (EC) function is mediated by variable hemodynamic shear stress patterns at the vascular wall, where complex shear stress profiles directly correlate with blood flow conditions that vary temporally based on metabolic demand. The interactions of these more complex and variable shear fields with EC have not been represented in hemodynamic flow models. We hypothesized that EC exposed to pulsatile shear stress that changes in magnitude and duration, modeled directly from real-time physiological variations in heart rate, would elicit phenotypic changes as relevant to their critical roles in thrombosis, hemostasis, and inflammation. Here we designed a physiological flow (PF) model based on short-term temporal changes in blood flow observed in vivo and compared it to static culture and steady flow (SF) at a fixed pulse frequency of 1.3 Hz. Results show significant changes in gene regulation as a function of temporally variable flow, indicating a reduced wound phenotype more representative of quiescence. EC cultured under PF exhibited significantly higher endothelial nitric oxide synthase (eNOS) activity (PF: 176.0 ± 11.9 nmol/10^5 EC; SF: 115.0 ± 12.5 nmol/10^5 EC, p = 0.002) and lower TNF-α-induced HL-60 leukocyte adhesion (PF: 37 ± 6 HL-60 cells/mm²; SF: 111 ± 18 HL-60/mm², p = 0.003) than cells cultured under SF which is consistent with a more quiescent anti-inflammatory and anti-thrombotic phenotype. In vitro models have become increasingly adept at mimicking natural physiology and in doing so have clarified the importance of both chemical and physical cues that drive cell function. These data illustrate that the variability in metabolic demand and subsequent changes in perfusion resulting in constantly variable shear stress plays a key role in EC function that has not previously been described.

Introduction
Vascular endothelial cells (EC) line the interior surface of blood vessels, providing a non-thrombogenic and selectively permeable barrier to circulating cells and macromolecules. EC are directly exposed to hemodynamic shear stress (SS), the frictional force applied by blood flow, and this stimulus is a principal mediator of EC phenotype.[1,2] Acute changes in blood flow patterns, which occur in response to variations in cardiac output/downstream metabolic demand, also change the patterns of SS applied, thereby eliciting phenotypic adaptations (e.g. changes in gene transcription/protein expression) in EC.

It is has previously been demonstrated using in vitro SS-generating culture systems that EC behave significantly differently under SS than they do under static conditions. Applied SS causes changes in gene transcription (up/downregulation) as well as protein expression/function.[1,2] Short-term adaptive changes to acute increases in SS (i.e. physiological increases in blood flow) include morphological reorientation of the cytoskeleton[3,4] and intracellular protein localization[5], and stimulation of enzymatic activity.[6,7] SS also stimulates metabolic production of endothelial-derived paracrine factors that regulate the physiology of both cells of the vascular wall (e.g. smooth muscle cells/fibroblasts) as well as those in the circulation (e.g. platelets, leukocytes, and stem cells).[1,7]

In vivo, the SS waveform to which the endothelium is exposed is dependent on blood flow conditions that vary by cardiovascular load, downstream metabolic demand, and local vascular geometry. As a result, EC phenotype is spatially heterogeneous throughout the vasculature.[8] Supporting clinical evidence exists in the focal development of atherosclerotic lesions in areas of the vasculature that experience disturbed (e.g. oscillatory or reversing) blood flow patterns, which have been linked to endothelial dysfunction.[9–13] The focal development of cardiovascular disease states in areas of the vascular wall exposed to disturbed blood flow underscores the sensitivity of the endothelium to variations in applied SS patterns.

To better understand the molecular signals underlying these phenotypic discrepancies, a number of useful computational models have been developed to recreate in vitro the atheroprotective/atherogenic SS profiles to which EC are exposed in various locations throughout vascular wall.[14–16] The adaptation of EC to deleterious SS patterns, such as shear gradients or flow oscillation, has been characterized by increased expression of atherogenic transcription factors, such as NF-kB, leading to a sustained pro-inflammatory state.[14,15,17–19] In contrast, exposure of EC to unidirectional, laminar flow downregulates
Physiological Shear Induces Endothelial Quiescence

inflammatory cell adhesion molecules and cytokines, and increases production of relaxing factors such as NO that inhibit cell adhesion, migration, and proliferation.[13,16,19]

An equally important consideration in the regulation of EC phenotype by hemodynamic SS is the dynamic nature of blood flow rate with respect to temporal demand. In vivo, homeostatic changes in cardiac output vary blood flow and pulse frequency to meet metabolic demand (e.g., during exercise), and so SS patterns at the blood-vascular endothelium interface is dynamic and in a constant state of change.[20] Local hemodynamic shear patterns, whether atherogenic or atheroprotective, are therefore not fixed mechanical stimuli but highly dynamic in terms of magnitude, amplitude, and duration.

Here we tested the hypothesis that temporal changes in applied SS patterns have an important influence on EC phenotypic expression. In this study, we designed an in vitro model of physiological flow primarily intended to mechanically stimulate EC across a variable range of SS, rather than a fixed or steady-state stimulus, which has been common in most model systems. Results highlight the significant phenotypic differences between primary human EC cultured under temporally modulated and steady pulsatile flow in vitro as relevant to their critical roles in thrombosis, hemostasis, and inflammation.

Materials and Methods

Ethics statement

Experiments involved de-identified human tissue samples were approved according to the Institutional Review Board-01 (Gainesville, FL; IRB approval #64-2010). Because tissue samples were indirectly obtained, and de-identified prior to collection, informed consent was not deemed necessary by the Institutional Review Board.

Endothelial cell isolation and expansion

Human umbilical cords were obtained from Labor & Delivery at Shands Hospital at the University of Florida (Gainesville, FL) and processed within 12 hours of delivery (IRB approval #64-2010). Human umbilical vein endothelial cells (EC) were isolated using collagenase perfusion as previously described.[21] Primary EC from three donors were pooled to reduce phenotypic variance. EC were maintained with VascuLife VEGF culture medium (LifeLine Cell Technologies) supplemented with 100 U/mL penicillin/streptomycin (HyClone), passaged every 2–3 days, and used experimentally between passages 2–4.

HL-60 cell culture

HL-60 promyelocytic leukemia cells (ATCC) transduced with a green fluorescent protein-expressing lentiviral vector were generously provided by Dr. Christopher Cogle (University of Florida, Gainesville, FL). They were maintained at concentrations between 5x10⁵ and 2x10⁶ cells/mL in Dulbecco’s Modified Eagle Medium supplemented with 20% FBS and 100 U/mL penicillin/streptomycin. Media was replenished every 2 days.

Endothelial cell perfusion culture

EC were seeded onto glass cover slips and allowed to grow to confluence over 48 hours before initiating flow. Monolayers were affixed to parallel plate flow chambers using a vacuum pump and connected to a media reservoir fitted with a 0.22 micron air filter for gas exchange. The entire system was placed in a dehumidified incubator maintained at 37°C and 5% CO₂. Masterflex Linkable Instrument Control Software V3.1 was used to control digital peristaltic pump drives (Masterflex) that generated pulsatile flow of media through each chamber (see Fig. 1). The mean wall SS (T) to which EC monolayers were exposed was calculated according to the Hagen-Poiseuille equation (assuming steady flow):

\[ T = \frac{4vQ}{\pi bh^2} \]

where v is media viscosity, Q is mean volumetric flow rate, and b and h are the base width and channel height, respectively. Mean volumetric flow was measured empirically, and system pressure was monitored using TruWave pressure transducers with Sonometrics SonoLAB digital acquisition system.

qPCR

After 24 hours, EC were collected and mRNA was purified using the RNAqueous–4PCR Kit (Ambion) according to instructions. mRNA was then normalized to an equivalent amount (400 ng per sample) and reverse-transcribed to cDNA using the RT² First Strand Kit (SABiosciences) according to instructions. cDNA was combined with RT² SYBR Green qPCR Master Mix and loaded onto human EC biology PCR arrays (SABiosciences). Amplification was performed at 95°C for 10 minutes, followed by 40 cycles of (95°C for 15 seconds and 60°C for 60 seconds). The comparative Cₜ method was used to quantify gene expression relative to the housekeeping genes GAPDH, RPL13A, B2M, ACTB, and HRP; EC cultured under static conditions were used as calibrating samples. Gene expression was reported as fold changes relative to calibrating samples; downregulated genes were reported inversely as negative fold changes.

Immunocytochemistry

After conditioning, EC were gently rinsed in PBS, formalin-fixed, and co-stained using rhodamine phalloidin/DAPI (Invitrogen). Images were obtained with a Zeiss AxioImager M2 fluorescence microscope using a Zeiss AxioCam Hrm Rev 3 digital camera operated by AxioVision 4.8.

Figure 1. Parallel plate culture system. Endothelial cell monolayers were grown to confluence on glass cover slips, then affixed to parallel plate flow chambers (A) using a vacuum pump. Peristaltic pumps (B) are used to impose pulsatile flow of culture media (C) over the endothelial monolayers. The rotational speed of the pumps is controlled by an external computer (D) via RS-232 linkage. doi:10.1371/journal.pone.0057004.g001
Nitrate/nitrite quantification

Total nitrate/nitrite content (stable salt derivatives of nitric oxide) in conditioned media was quantified using the Nitrate/Nitrite Fluorometric Assay Kit (Cayman Chemical). 4-6 replicate samples were analyzed per flow condition, and results were normalized by cell number.

HL-60 cell adhesion assay

Monolayers were activated during the final four hours of flow with 1 unit (0.16 ng/mL) recombinant human TNF-α (Thermo Scientific). At hour 24, cover slips were removed from flow circuits, rinsed in media, and incubated with a bolus (10⁶ cells/mL) of GFP-expressing HL-60 cells within Petri dishes for 10 minutes. Monolayers were rinsed 3 times in PBS and stained as described above. The number of adherent HL-60 cells were quantified in 15 specified locations throughout the flow field for each condition (n = 6).

Statistical analysis

Results are presented as mean±SEM. One-way ANOVA followed by post-hoc Tukey-Kramer HSD analyses (with the significance level set at 0.05) were conducted to compare gene expression by experimental group or NO production by group or time. Alternatively, when equal variances could not be assumed, NO production by group was compared using Tamhane’s T2 test. Student’s t-test (significance set at 0.05) was used to compare HL-60 cell adhesion.

Results

We developed a flow regime that mimics constant physiological variability associated with cardiac output to mechanically stimulate endothelial monolayers across a physiological range of arterial shear stress (SS). Real-time recordings of human heart rate in a healthy male subject were obtained over a single 12-hour period and programmed into computer-driven peristaltic pump drives so that rotational speed corresponded to the observed changes in cardiac output. The calculated mean wall SS within parallel plate flow chambers downstream of the pumps (see Fig. 2) ranged from a minimum 7.2 dynes/cm² at 56 pulses/min to a maximum 18.4 dynes/cm² at 142 pulses/min. For comparison, steady pulsatile flow regimes applied the same total magnitude of SS, albeit at different rates. Therefore, both flow regimes induced a model flow program averaged over the entire 12 hour cycle. Thus, both flow regimes applied the same total magnitude of SS, albeit at different rates.

Physiologically modeled flow induces morphological adaptation in endothelial cells

Primary human endothelial cell (EC) monolayers were grown to confluence on glass cover slips and cultured under one of three experimental conditions for 24 hours: static culture, steady flow (SF), or physiological flow (PF). For PF, two cycles were applied back-to-back to normalize the 24 hour perfusion culture period. EC maintained under static culture conditions grew to a higher density than those cultured under flow, with no global cytoskeletal organization (see Fig. 3A). As expected, culturing monolayers under laminar flow (SF or PF) induced alignment of cytoskeletal F-actin fibers in the flow direction (see Fig. 3B, C). No significant global differences in cytoskeletal morphology were apparent between EC cultured under SF or PF after 24 hours, indicating similar adaptation to both shear regimes.

Physiological flow induces cardio-protective gene expression in endothelial cells

After 24 hours of conditioning under static culture, SF, or PF, EC mRNA was extracted, reverse-transcribed to cDNA, and amplified using qPCR. We assessed expression of several EC genes which, due to their importance in vascular physiology, are standard clinical diagnostic markers of cardiovascular health or disease (see Fig. 4). Maintaining EC under 24 hours of either SF or PF resulted in upregulation of superoxide dismutase-1 (SOD-1), an enzyme that protects against oxidative stress (SF: 1.493±0.064-fold, p = 0.001; PF: 1.495±0.065-fold, p = 0.001). Furthermore, we observed the vasopressor gene endothelin-1 (ET-1), which causes smooth muscle cell contraction and vasoconstriction and is implicated in atherosclerosis[22], was downregulated by both types of flow (SF: −7.710±0.999-fold, p = 0.002; PF: −1.775±0.258-fold, p = 0.048), with no significant difference between flow groups (p = 0.053). EC expression of prostacyclin synthase, a potent vasodilator and inhibitor of platelet aggregation, and characteristic marker of endothelial quiescence, was dependent on the flow regime imposed. Interestingly, PTGS was dramatically downregulated by SF (−4.493±0.089-fold, p = 0.004), but unaffected by PF (1.032±0.104-fold, p = 0.171).

Physiological flow does not significantly modulate expression of coagulation/fibrinolysis genes in endothelial cells

Distinct expression trends were found in EC genes associated with coagulation and fibrinolysis (see Fig. 5) between static culture and SF. Annexin A5 (ANXA5), a plasma protein with anticoagulant properties, was upregulated by SF (1.429±0.089-fold, p = 0.015), but not significantly affected by PF (1.317±0.073-fold, p = 0.062). Anti-clotting proteins tissue factor pathway inhibitor (TFFP) and thrombomodulin (THBD) did not significantly vary by experimental group. Fibrinolytic enzyme urokinase plasminogen activator (PLAU) was upregulated by SF alone (1.844±0.223-fold, p = 0.031), and plasminogen activator inhibitor (PAI-1) expression was significantly higher in cells conditioned under PF than SF (1.541±0.227-fold vs. −1.411±0.145-fold, p = 0.034), though neither significantly deviated from static culture. Overall, SF lowered expression of procoagulant genes and increased expression of fibrinolytic proteins, while PF evoked similar trends, none significantly deviated from static culture.

Physiological flow induces higher endothelial cell expression of chemotactic factors than steady flow

EC expression of genes associated with inflammation varied greatly depending on flow conditions (see Fig. 6A,B). Culture under SF resulted in upregulation of the cell adhesion molecules ICAM-1 (4.467±0.660-fold, p = 0.006) and PECAM-1 (3.180±0.152-fold, p = 0.000), and downregulation of VCAM-1 (−9.104±0.116-fold, p = 0.006) compared with static culture. No PF-conditioned EC adhesion molecules significantly varied in expression relative to static controls, though PECAM-1 expression was significantly lower (1.566±0.196-fold, p = 0.000) when compared to steady flow.

Expression of monocyte chemoattractant protein-1 (MCP-1) was significantly downregulated under either flow condition, though significantly more so in EC conditioned under SF (−9.620±1.352-fold) than PF (−2.715±0.246-fold, p = 0.049). Fractalkine (CX3CL1) was upregulated in PF-conditioned cells only (2.522±0.437-fold, p = 0.028), and was significantly higher than in cells cultured under SF (−1.882±0.611-fold, p = 0.004). ADAM17, an enzyme that mediates TNF-a shedding from the
Physiological Shear Induces Endothelial Quiescence

Figure 2. Physiologically modeled perfusion culture. A 12-hour perfusion regime (hollow line) was developed by programming a series of real-time recordings in heart rate obtained in a healthy male subject into peristaltic pumps as ramp changes. For comparison, the average shear stress magnitude calculated over the entire 12-hour cycle (10.3 dynes/cm² at 80 pulses/min) was programmed as steady pulsatile flow (dark line). Mean wall shear stress and pulse frequency experienced by the endothelial cells within parallel plate flow chamber over time are shown on the left and right axes, respectively. Insets show pressure waveforms experienced at various time points during steady (dark border) or physiological flow (hollow border).

doi:10.1371/journal.pone.0057004.g002

Physiological flow induces sustained endothelial nitric oxide synthase activity

Endothelial nitric oxide synthase (eNOS) converts L-arginine to L-citrulline and nitric oxide, the latter of which is an important inhibitor of leukocyte adhesion, platelet aggregation, and smooth muscle proliferation. This enzyme is constitutively expressed, yet highly regulated in EC.[23,24] Nitric oxide is an important signaling molecule in vascular physiology, as its inhibition of

Figure 3. Cytoskeletal morphology of flow-conditioned endothelial cells. Endothelial cell monolayers were grown to confluence and cultured under static conditions (A), steady flow (B), or physiological flow (C) for 24 hours. Monolayers were subsequently fixed and co-stained with rhodamine phalloidin (middle row) and DAPI (top row) in order to visualize f-actin and cell nuclei, respectively. Shown are representative images (40x) from each condition. Applying shear stress resulted in cytoskeletal alignment of endothelial cells in the flow direction (horizontally left to right). Scale bar: 20 microns.

doi:10.1371/journal.pone.0057004.g003

Figure 4. Cardio-protective gene expression in flow-conditioned endothelial cells. Shown is fold mRNA expression (with respect to static-cultured EC) of genes that promote or inhibit cardiovascular disease progression. Results are presented as mean±SEM. An embedded asterisk indicates a significant difference with respect to static controls; an asterisk over a bracket indicates a significant difference between flow groups. Abbreviations: EDN1: endothelin-1; PTGIS: prostacyclin synthase; SOD1: superoxide dismutase-1.

doi:10.1371/journal.pone.0057004.g004
Leukocyte adhesion and platelet aggregation facilitates laminar, uninterrupted blood flow. EC expression of eNOS mRNA was significantly upregulated after 24 hours of flow conditioning (SF: 2.049 ± 0.086-fold, p = 0.000; PF: 1.715 ± 0.069-fold, p = 0.003, see Fig. 7A). However, there was no significant difference in eNOS gene expression between the two flow groups (p = 0.076). eNOS enzymatic activity was assessed by quantifying the total amount of nitrates/nitrites, stable salt derivatives of nitric oxide, in conditioned culture media after 0, 12, or 24 hours of culture and normalized by EC number (see Fig. 7B). Under static culture conditions, NO content did not significantly vary by culture time: (0 hours: 38.745 ± 8.144 nmol/10^5 EC, 12 hours: 41.552 ± 10.308 nmol/10^5 EC, 24 hours: 33.680 ± 2.614 nmol/10^5 EC, p = 0.689). After 12 hours of perfusion culture, significant increases in NO byproduct accumulation were observed (SF: 88.308 ± 7.608 nmol/10^5 EC, p = 0.025; MF: 84.993 ± 11.233 nmol/10^5 EC, p = 0.030), with no significant difference in means observed between flow groups (p = 0.969).

However, noteworthy deviations in NO output occurred between flow regimes during the last 12 hours of perfusion. No significant increase in nitrates/nitrites was observed between 12 and 24 hours of SF (12 hours: 88.308 ± 7.608 nmol/10^5 EC, 24 hours: 115.039 ± 12.460 nmol/10^5 EC, p = 0.186). PF, however, induced significant temporal increases in NO output between 12 (84.993 ± 11.233 nanomoles/10^5 EC) and 24 hours (176.0 ± 11.860 nanomoles/10^5 EC, p = 0.000) of conditioning. After 24 hours, the highest nitrate/nitrite concentrations were measured in media from EC cultured under PF, which was significantly higher than that from EC cultured under SF (p = 0.002).

Nitric oxide production by endothelial cells is significantly higher with programmed shear changes than constant-frequency flow

For added clarity, two additional flow groups were included to profile nitric oxide production under physiologically dynamic conditions. First, another steady flow group (SF-160) was included in which the flow was applied at twice the rate of the SF (160...
Physiological flow enhances endothelial cell resistance to activation-induced leukocyte adhesion

Given the dramatic increase in NO production noted in PF-conditioned EC compared with steady flow, we next performed a functional assay to characterize the extent to which temporal changes in shear influenced EC adhesiveness for leukocytes. After four hours of activation with TNF-α, flow-conditioned monolayers were transferred to Petri dishes and incubated with a bolus of promyelocytic GFP<sup>+</sup> HL-60 cells (see Fig. 8). Conditioning EC under PF significantly reduced HL-60 leukocyte attachment compared to conditioning under SF (PF: 37 ± 6 HL-60 cells/mm<sup>2</sup>; SF: 111 ± 18 HL-60/mm<sup>2</sup>, p = 0.003).
Discussion

Vascular endothelial cell (EC) phenotype is a function of multiple physiological factors, of which appropriate shear stimulation is critical for maintaining EC in a quiescent, non-thrombogenic state. Defining how hemodynamic shear patterns mediate EC function, as well as pathological dysfunction, is critical to our understanding of vascular physiology.[9,11,25,26]

In vitro systems designed to expose EC to controlled levels of fluid shear stress (SS) have greatly advanced our understanding of the molecular mechanisms underlying the transduction of hemodynamic shear stress into biological signals.[2,27] It is now recognized that specific details of the shear waveform have important implications for EC adaptation/functionality. For example, EC morphology, gene expression, enzymatic activity have all been shown to vary depending on whether flow is applied steadily or with pulsation, as occurs in vivo.[15,28,29] It has become increasingly clear that recapitulating the complex characteristics of physiological blood flow is critical to the utility of in vitro model systems.

A significant gap lies between the majority of in vitro perfusion culture systems and physiological blood flow patterns seen in vivo.[25] Although experimental flow models accurately recreate the shear waveforms experienced at specific locations on the vascular wall[14–16], the waveform is most often applied cyclically, without modulation of frequency or amplitude for the duration of the experiment. The temporal variations in blood flow rate that continuously occur in vivo are not taken into account. Given that EC phenotype has been shown to be a function of pulse frequency[30], SS magnitude[31,32], and pulse amplitude[33], all of which vary in vivo throughout the course of the day, blood flow dynamics warrant consideration in these systems.

Here we tested the hypothesis that temporal variability in applied SS would induce changes to EC phenotype as relevant to their critical roles in circulatory/vascular biology. The physiological flow (PF) model in this study was developed to simulate physiological changes in pulse rate and volumetric flow rate as they occur in vivo over the course of a single day. The model encompassed a range of SS commonly found in small diameter arteries.[34–36] Steady flow with an equivalent time-averaged SS and fixed pulse frequency was used to represent the conditions used in current systems.

Transcriptional profiling of EC exposed to steady pulse frequencies versus physiologically modeled flow demonstrated significant differences in gene expression. Prostacyclin synthase, the enzyme responsible for production of a potent vasodilator/inhibitor of platelet aggregation, was significantly downregulated in cells conditioned under steady flow, while no change was induced by physiological flow. The leukocyte adhesion molecule/chemokine fractalkine was upregulated under physiological flow alone. In some cases, gene expression trends (relative to static-cultured cells) were statistically similar regardless of flow regime (protective enzymes eNOS, SOD-1 were upregulated; leukocyte chemokine MCP-1 was downregulated). Elucidating the various signaling mechanisms involved in gene transcription regulation by SS is an area of intense interest. The transcription factor KLF-2, for example, has been found to play a role in eliciting the atheroprotective effects of laminar SS in EC, both through induction of eNOS expression as well as inhibition of agonist-induced expression of E-selectin and VCAM-1.[18] Boon et al have published a review on several known SS-responsive transcription factors[37], and the role of these factors in the presently observed results will be the topic of future investigations. Because only endpoint gene expression analysis was performed, temporal trends were not observed and comparisons between the PF model and the steady control at 24 hours alone limits the number of conclusions that may be drawn. Taken collectively, however, the data show a more quiescent EC transcriptome elicited by both flow regimes compared to statically cultured cells.

Shear stimulation of EC has important implications for metabolic production of nitric oxide (NO), an important inhibitor of platelet aggregation[30], leukocyte adhesion[39], and smooth muscle cell mitogenesis[40]. Diminished NO bioavailability is a hallmark of endothelial dysfunction, which is a prevalent symptom in a wide variety of cardiovascular disorders, including thrombosis[41], intimal hyperplasia[42], pulmonary hypertension[43], and atherosclerosis.[44] In the present study, notable increases in nitric oxide (NO) production were observed when EC were exposed to PF, a regime characterized by temporal increases (and decreases) in SS magnitude/pulse frequency. In agreement with previous studies, we found that application of SS caused upregulation of eNOS gene expression.[28,45] Though eNOS expression between cells exposed to each flow regime did not statistically vary, the metabolic activity of the enzyme changed dramatically. While endothelial NO production tapered off after 12 hours of steady flow, physiological flow induced dramatic increases in NO synthesis between 12 and 24 hours.

Previous research has shown that endothelial NO production is dependent on not only the overall magnitude but also the rate at which shear stimulation is applied.[6,28,46] While rapid increases in SS induce a transient G protein coupled receptor-dependent burst in NO production, steady flow results in a state of sustained NO production.[47] We postulate that the tapering off in NO output is due to physiological adaptation of EC to the steady-state conditions associated with fixed-pulse flow. SS modulates eNOS activity in EC through a variety of mechanisms: calmodulin binding, phosphorylation state of certain residues, and association with membrane-bound proteins such as PECAM-1.[23,48,49] In particular, acute SS increases have been shown to elevate eNOS activity through phosphorylation of Ser1177 residue by PI-3K.[50] The rapid increases in shear within the physiological flow regime likely stimulated periodic bursts in NO production that were not induced under steady flow. The significantly lowered leukocyte adhesiveness in physiological flow-conditioned EC observed correlates with higher NO production, as well as significantly lower expression of PECAM-1 when compared to steady flow.[51]

The present evidence suggests EC quiescence can be induced by dynamic stimulation across a physiological range of arterial shear stresses. It is therefore plausible to consider that temporal alterations in mechanical stimulation may affect functionality of other cell types as well. Sydelcain et al previously demonstrated increased ERK signaling and greater collagen synthesis by human dermal fibroblasts cultured in a cyclic distension bioreactor when incremental increases in stretch were applied, rather than fixed distension.[52] Though disparities exist between the two model systems, both show evidence that the response of cells to mechanical stimulation is dependent on the overall magnitude/frequency of the stimulus. Application of variable mechanical stimuli to cells cultured in other bioreactor systems that may influence regeneration of blood vessels, tendon, bone, cartilage, or other tissues in vivo.

In conclusion, we have demonstrated the inherent sensitivity of critical EC functions to temporal changes in applied SS. The steady state flow applied in current culture systems, while useful for close examination of molecular signaling events, may not accurately represent the physiological hemodynamics to which EC are exposed in vivo. These investigations show a clear EC...
phenotype modulation toward a quiescent arterial state and improved functionality in a simulated wound environment. Understanding the conditions that regulate EC function and having the capacity to model these appropriately in vitro has significant implications for treatment of clinical pathologies associated with endothelial dysfunction.

Supporting Information

Figure S1 Nitric oxide profiling of endothelial cells having the capacity to model these appropriately in vitro improved functionality in a simulated wound environment. Phenotype modulation toward a quiescent arterial state and associated with endothelial dysfunction.

References

1. Chien S (2007) Mechanotransduction and endothelial cell homeostasis: the wisdom of the cell. Am J Physiol Heart Circ Physiol 292: H209–224.
2. Davies PF (1995) Flow-mediated endothelial mechanotransduction. Physiol Rev 75: 519–560.
3. Roullet L, Farca M, Tardif JC, Mougrain R, Leak RL (2010) Endothelial cell morphologic response to asymmetric stenosis hemodynamics: effects of spatial wall shear stress gradients. J Biomech Eng 132: 081013.
4. Dewey CF, Busolari SR, Gimbrone MA, Davies PF (1981) The dynamic response of vascular endothelial cells to fluid shear stress. J Biomech Eng 103: 177–185.
5. Sakamoto N, Segawa K, Kanzaki M, Ohashi T, Sato M (2010) Role of p120-catenin in the morphological changes of endothelial cells exposed to fluid shear stress. Biochem Biophys Res Commun 390: 436–442.
6. Butler PJ, Weinbaum S, Chien S, Levenson DE (2000) Endothelium-dependent, shear-induced vasodilation is rate-sensitive. Microcirculation 7: 53–65.
7. Frangos JA, McIntire LV, Eskin SG (1989) Shear stress induced stimulation of mammalian cell proliferation. Biotechnol Bioeng 32: 1053–1060.
8. Chj JT, Chang HY, Haraldsen G, Troyanskaia OG, et al. (2003) Endothelial cell diversity revealed by global expression profiling. Proc Natl Acad Sci U S A 100: 10623–10628.
9. Davies PF (2009) Hemodynamic shear stress and the endothelium in cardiovascular pathophysiologic. Nat Clin Pract Cardiovasc Med 6: 16–26.
10. Caro CG, Fitz-Gerald JM, Schroter RC (1969) Arterial wall shear and distribution of early atheroma in man. Nature 222: 1159–1160.
11. Gimbrone MA, Topper JN, Nagel T, Anderson KR, Garcia-Cardena G (2000) Endothelial dysfunction, hemodynamic forces, and atherogenesis. Ann N Y Acad Sci 902: 230–239; discussion 239–240.
12. Davies PF (2008) Endothelial transcriptome profiles in vivo in complex arterial flow fields. Ann Biomed Eng 36: 563–570.
13. Gimbrone MA (1999) Vascular endothelium, hemodynamic forces, and atherogenesis. Ann N Y Acad Sci 902: 230–239; discussion 239–240.
14. Dai G, Kazaemzadeh-Moat MR, Natarajan S, Zhang Y, Vaughn S, et al. (2004) Distinct endothelial phenotypes evoked by arterial waveforms derived from atherosclerosis-susceptible and -resistant regions of human vasculature. Proc Natl Acad Sci U S A 101: 14871–14876.
15. Blackman BR, Garcia-Cardeña G, Gimbrone MA (2002) A new in vitro model to evaluate differential responses of endothelial cells to simulated arterial shear stress waveforms. J Biomech Eng 124: 397–407.
16. Wang N, Mao H, Li YS, Zhang P, Haga JH, et al. (2006) Shear stress regulation of Krippelt-factor 2 expression is flow pattern-specific. Biochem Biophys Res Commun 341: 1244–1251.
17. Hahn C, Schwartz MA (2008) The role of cellular adaptation to mechanical forces in atherosclerosis. Arterioscler Thromb Vasc Biol 28: 2108–2114.
18. SenBanerjee S, Lin Z, Atkins GB, Greif DM, Rao RM, et al. (2004) KLF2 Is a transcriptional and posttranscriptional regulator of vascular endothelial and smooth muscle cell gene expression. J Exp Med 199: 1305–1317.
19. Dai G, Vaughn S, Zhang Y, Wang ET, Garcia-Cardenas G, et al. (2007) Biomechanical forces in atherosclerosis-resistant vascular regions regulate endothelial redox balance via phosphoinosit 3-kinase/Akt-dependent activation of Nrf2. Circ Res 101: 723–733.
20. Schulze EL, Schullmacher S, Minzel T (2009) When metabolism rules perfusion. AMPK-mediated endothelial nitric oxide synthase activation. Circ Res 104: 422–424.
21. Jaffe EA, Nachman RL, Becker CG, Minick CR (1973) Cultures of human endothelial cells derived from umbilical veins. Identification by morphology and immunologic criteria. J Clin Invest 52: 2747–2756.
22. Thorsen E, Webb DJ (2010) Endothelium-derived endothelin-1. Physiologist 53: 951–958.
23. Mount PF, Kemp BE, Power DA (2007) Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation. J Mol Cell Cardiol 42: 271–279.
24. Searles CD (2006) Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression. Am J Physiol Cell Physiol 291: C1303–1316.
25. Newcomer SC, Thijssen DH, Green DJ (2011) Effects of exercise on endothelium and endothelium/smooth muscle cross-talk: role of exercise-induced hemodynamics. J Appl Physiol 111: 311–320.
26. Neren RM, Levesque MJ (1983) Fluid dynamics as a factor in the localization of atherogenesis. Ann N Y Acad Sci 416: 709–719.
27. White CR, Frangos JA (2007) The shear stress of it all: the cell membrane and mechanoskeletal transduction. Plilos Trans R Soc Lond B Biol Sci 362: 1459–1467.
28. Yee A, Bosworth KA, Conway DE, Eskin SG, McIntire LV (2008) Gene expression of endothelial cells under pulsatile non-reversing vs. steady shear stress: comparison of nitric oxide production. Ann Biomed Eng 36: 571–579.
29. Deldr RF, van Thienen JV, Roldena J, de Jager SC, Elderkamp CV, et al. (2005) Endothelial KLF2 links local arterial shear stress levels to the expression of vascular tone-regulating genes. Am J Pathol 167: 689–691.
30. Himburg HA, Dowell SE, Friedman MH (2009) Frequency-dependent response of the vascular endothelium to pulsatile stress. Am J Physiol Heart Circ Physiol 293: H654–653.
31. Rouleau L, Ross J, Leak RL (2010) The response of human aortic endothelial cells in a stenotic hemodynamic environment: effect of orientation, magnitude, and spatial gradients in wall shear stress. J Biomech Eng 132: 071013.
32. Ranjan V, Xiao Z, Diamond SL (1995) Constitutive NO expression in cultured endothelial cells is elevated by fluid shear stress. Am J Physiol 269: H550–555.
33. Li M, Scott DE, Sandals R, Stenmark KB, Tan W (2009) High pulsatility flow induces adhesion molecule and cytokine mRNA expression in distal pulmonary artery endothelial cells. Ann Biomed Eng 37: 1092–1092.
34. Papaioannou TG, Karatzis EN, Vavuranakis M, Lekakis JP, Stefanadis C (2006) Assessment of vascular wall shear stress and implications for atherosclerotic disease. Int J Cardiovasc Ther 13: 211–222.
35. Reneman RS, Hoeks AP (2008) Wall shear stress as measured in vivo: consequences for the design of the arterial system. Med Biol Eng Comput 46: 499–507.
36. Dorost PA, Dorsaz PA, Dorazl I, De Beusteri E, Chateiain P, et al. (2000) In vivo measurements of wall shear stress in human coronary arteries. Coron Artery Dis 11: 495–502.
37. Boom RA, Horrevoets AJ (2009) Key transcriptional regulators of the vasoprotective effects of shear stress. Hum Genet 124: 39–40.
38. Furlong B, Henderson AH, Lewis MJ, Smith JA (1987) Endothelium-derived relaxing factor inhibitor is in vitro platelet aggregation. Br J Pharmacol 90: 617–692.
39. Kubas P, Suzuki M, Granger DN (1991) Nitric oxide: an endogenous modulator of platelet aggregation. Proc Natl Acad Sci U S A 88: 4651–4653.
40. Gang UC, Hassid A (1989) Nitric oxide-generating vasodilators and 1H-bromocyclo guanidine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. J Clin Invest 83: 1774–1777.
41. Wu K, Thiegarajan P (1996) Role of endothium in thrombosis and hemostasis. Annu Rev Med 47: 315–331.
42. Atef MJ, Tzeng E, Zuckerman BS (2012) Nitric oxide and nitric-based therapeutic opportunities in intimal hyperplasia. Nitric Oxide.
43. Sparacino-Watkins GE, Lai YC, Gladwin MT (2012) Nitrate- Nitrite- Nitric Oxide Pathway in Pulmonary Arterial Hypertension Therapeutics. Circulation.
44. Naseem KM (2005) The role of nitric oxide in cardiovascular diseases. Mol Med 19: 1205–1331.
and sustained nitric oxide production. Biochem Biophys Res Commun 224: 660–665.

47. Kuchan MJ, Jo H, Frangos JA (1994) Role of G proteins in shear stress-mediated nitric oxide production by endothelial cells. Am J Physiol 267: C753–758.

48. Michel T, Vanhoutte PM (2010) Cellular signaling and NO production. Pflugers Arch 459: 807–816.

49. Bagi Z, Frangos JA, Yeh JC, White CR, Kaley G, et al. (2005) PECAM-1 mediates NO-dependent dilation of arterioles to high temporal gradients of shear stress. Arterioscler Thromb Vasc Biol 25: 1590–1595.

50. Li Y, Zheng J, Bird IM, Magness RR (2004) Mechanisms of shear stress-induced endothelial nitric-oxide synthase phosphorylation and expression in ovine fetoplacental artery endothelial cells. Biol Reprod 70: 783–796.

51. Lindemann S, Sharafi M, Sprecker M, Buerke M, Fisch A, et al. (2000) NO reduces PMN adhesion to human vascular endothelial cells due to downregulation of ICAM-1 mRNA and surface expression. Thromb Res 97: 113–123.

52. Syedain ZH, Weinberg JS, Tranquillo RT (2008) Cyclic distension of fibrin-based tissue constructs: evidence of adaptation during growth of engineered connective tissue. Proc Natl Acad Sci U S A 105: 6537–6542.