Nitric oxide-dependent stimulation of endothelial cell proliferation by sustained high flow

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Metaxas E, Meng H, Kaluvala SR, Szymanski MP, Paluch RA, Kolega J. Nitric oxide-dependent stimulation of endothelial cell proliferation by sustained high flow. Am J Physiol Heart Circ Physiol 295: H736–H742, 2008. First published June 13, 2008; doi:10.1152/ajpheart.01156.2007.—Little is understood about endothelial cell (EC) responses to high flow, which mediate adaptive outward remodeling as well as cerebral aneurysm development. Opposite EC behaviors have been reported in vivo including cell loss during aneurysm initiation and cell proliferation during adaptive outward remodeling. This study aims at elucidating the EC growth response to elevated wall shear stress (WSS) and determining if nitric oxide (NO) is involved. A confluent EC monolayer was subjected to steady-state, laminar flow with WSS ranging from 15 to 100 dyn/cm² for 24 and 48 h. Cells oriented to the direction of the flow with a time course that varied with WSS. At 48 h, all cells were aligned with the flow. EC proliferation was examined using bromodeoxyuridine (BrdU) incorporation. The percentage of proliferating ECs rose linearly from 15 to 50 dyn/cm² to more than sixfold at 50–100 dyn/cm². The percentage of proliferating ECs rose linearly from 15 to 50 dyn/cm² to more than sixfold at 50–100 dyn/cm² compared with the accepted physiological baseline of 15–20 dyn/cm². Immunostaining revealed increased endothelial nitric oxide synthase (eNOS) production with increasing WSS. These results demonstrate that high WSS stimulates EC proliferation and suppresses apoptosis. Furthermore, immunostaining revealed increased endothelial nitric oxide synthase (eNOS) production with increasing WSS. NOS inhibition with N-nitro-l-arginine methyl ester (l-NAME) drastically reduced the WSS-stimulated proliferation, indicating a critical role of NO production in the stimulation of EC proliferation by high WSS.

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Although there is a wealth of knowledge about endothelial cell (EC) responses to low wall shear stress (WSS<15 dyn/cm²) because of its suspected role in atherogenesis (3, 10), very little is known about the effect of high WSS (>50 dyn/cm²) on EC function. Such high WSS hemodynamics occur during compensatory flow in collateral arteries secondary to arterial blockage (25, 29) and exist chronically at specific locations that are prone to intracranial aneurysm initiation such as the apex of a bifurcation or outer side of a curvature (16). Endothelial function under high WSS is important for vascular homeostasis or in the failure thereof.

The bulk of literature about EC function under flowing blood suggests that physiological levels of WSS (15–20 dyn/cm²) suppress EC proliferation compared with static or low WSS conditions (2, 7, 18, 21). However, chronically elevated WSS would be expected to stimulate EC proliferation to maintain endothelial cover of an enlarged wall. In rabbits with arteriovenous fistula, increased blood flow through the common carotid artery (and consequent elevation of WSS to ~80 dyn/cm²) caused adaptive outward arterial remodeling, which was accompanied by EC proliferation (24, 25). On the other hand, EC dysfunction [downregulation of endothelial nitric oxide synthase (eNOS)] has been observed during aneurysm initiation at bifurcations exposed to high WSS (14), and denudation or EC loss has been frequently noted as a characteristic of aneurysmal walls (8, 13, 15, 20).

These different EC behaviors at high flow environments may reflect in vivo complexity. The behavior of ECs in vivo is affected not only by WSS, but also by blood pressure, which is a major determinant of vessel stretch, and by paracrine signals from other vascular tissues (such as smooth muscle cells in the media). Therefore, it is difficult to sort out the direct effect of high WSS on ECs based on in vivo observations. In the absence of a fundamental understanding of how ECs respond to the high WSS and how this might be related to adaptive or maladaptive vascular remodeling such as aneurysm formation, this study aims at elucidating the EC growth response to elevated flow. It also examines the role of nitric oxide (NO) in this response, because both the suppression of EC proliferation by low WSS in vitro and the stimulation of vessel growth by high flow in vivo reportedly require endothelial NO production (4, 27).

MATERIALS AND METHODS

Flow loop. Bovine aortic endothelial cells (BAECs) were exposed to defined WSS levels by placement in a flow loop consisting of a reservoir, a peristaltic pump, two dampeners, and either a tapered chamber or a parallel chamber. To minimize spallation, Gore Sta-Pure tubing was used in the pump section. The flow rate was controlled with the pump speed, and the pressure was controlled with an adjustable clamp placed after the chamber, allowing variable constriction of the tubing to produce pressures ranging from 30 to 200 mmHg. An ultrasonic flow probe (Transonic Systems) was used to measure the flow rate, and a pressure transducer (Becton Dickinson) was placed just before the chamber to measure the pressure, which was set at 100 mmHg in all experiments.

Tapered chamber. To achieve a wide range of WSS values, a tapered chamber was designed. Its height was tapered along the flow direction in the test section to achieve gradually accelerating flow.
thereby increasing WSS monotonically along the distance (Fig. 1). To establish a fully developed laminar flow over the cells, the chamber had an entry length 10 times the cross-sectional height of the flow, upstream of the cell test section. ProEngineer (PTC) solid modeling was used to design smooth transitions from cylindrical tubing to rectangular chamber cross-sections to avoid flow disturbances. The tapered chamber was constructed of silicone elastomer (Sylgard), which was formed in specifically designed molds produced using rapid prototyping based on the ProEngineer geometry. The test section had a rectangular groove (22 × 50 mm²) for the placement of the microscope cover glass (Fisher Scientific, Pittsburgh, PA) with the attached cells. The height of the channel above the cover glass ranged from 0.5 to 0.16 cm.

**Characterization of WSS distribution.** Computational fluid dynamics (CFD) simulations were performed on the geometry of the tapered chamber to characterize the WSS distribution. As computational grids, tetrahedral and prism elements were generated with ICEM-CFD software (Ansys), and CFD solutions were obtained using Star-CD (CD-Adapco). The fluid properties, μ = 3.5 cP and density = 1,020 kg/m³, were obtained from measurements on the experimental working fluid. For steady-state flow conditions, the flow rate of 900 ml/min was found to produce the desired distribution of WSS values on the EC monolayer surface in the test section.

**Parallel chamber.** To validate the results of the tapered chamber, a commercially available parallel chamber was used (Streamer, Flexcell) to obtain uniform WSS of a specific value (85 dyn/cm²). To use coverslips of the same quality as those in the tapered chamber, we manufactured coverslip holders to replace the glass slides provided by the manufacturer. This modification did not alter the flow through the device.

**Culture preparation.** Early passage BAECs were cultured on microscope cover glasses with DMEM and 10% FBS for 4–6 days until they reached confluence. Cultures were equilibrated in flow media containing DMEM, 5% FBS, and 8% Dextran 70 (Sigma), which was used to match the viscosity of blood, for 24 h before exposure to flow. Time-matched static controls were maintained under the same conditions but were not exposed to flow. Each experiment was performed in triplicate to obtain redundancy.

**Cell proliferation assay.** Proliferating BAECs were identified by using a commercial in situ monoclonal antibody kit (Roche) for the detection of bromodeoxyuridine (BrdU) incorporation into cellular DNA. Two hours before stopping the experiment, BrdU labeling media was added in the loop. After 2 h in the presence of BrdU, cells were fixed in 70% ethanol (in 50 mmol/l glycine buffer, pH 2.0) and immunostained for BrdU incorporation followed by a fluorescein-conjugated secondary antibody and mounted with media containing diamidino-2-phenylindole dihydrochloride (DAPI) for staining nuclei.

**eNOS protein assay.** The EC monolayer was fixed with 10% formalin and stained for eNOS protein using an anti-eNOS monoclonal antibody (BD Biosciences) followed by a rhodamine-conjugated secondary antibody. After fixing the EC monolayer with 1% paraformaldehyde, terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) staining was performed using a TUNEL Apoptosis Detection Kit (Chemicon International).

**Imaging.** To spatially map the percentage of proliferating cells in regions of different WSS, a composite image of the entire length of the EC monolayer was created by stitching together sequential images of the cells taken at ×10 magnification. The edges of the coverslip (5 mm from each end) were excluded to eliminate areas of cell disruption due to handling. Images were acquired and assembled using a Zeiss Axio Imager motorized fluorescence microscope and accompanying Zeiss Axio Imager software. Positively stained cells were quantified using macros in NIH ImageJ (1). The macros divided the image into regions of interest (ROI) of 200 μm width by 2,000 μm height along the length of the slide. For each ROI, the total number of DAPI-positive cells and BrdU-positive or TUNEL-positive cells were counted. The DAPI and BrdU or TUNEL measurements were used to calculate cell density and the percentage of proliferating or apoptotic cells in each region. Density and proliferation or apoptosis data from 10 ROI were averaged for every 1 mm along the glass slide. Image analysis was also done to quantify the eNOS protein intensity, which was normalized by the cell number.

**NO inhibition.** In those experiments designed to test the effect of NO inhibition, the EC monolayer was pretreated for 2 h before exposure to flow with 1 mM Nω-nitro-L-arginine methyl ester (L-NAME), a nonspecific NOS inhibitor, and 1 mM L-NAME was included in the circulating media during the entire exposure (24 h) to flow.

**Statistical analysis.** Each experiment was performed at least three times, and all values are represented as means ± SD. Statistical analysis was performed using the SYSTAT statistical package (Richmond, CA). Mixed regression models were used to examine the effect of WSS magnitude on EC proliferation at 48 h and on apoptosis and eNOS expression at 24 h. The cell density, percentage of proliferating cells, percentage of apoptotic cells, and eNOS expression were each modeled as linear functions of WSS magnitude:

\[
Y = \beta_0 + \beta_1(WSS) + \varepsilon
\]

where \(\beta_0\) is the intercept treated as random, the slope, \(\beta_1\), reflects the degree of dependence of \(Y\) on WSS and is treated as fixed, and \(\varepsilon\) is the random error of the sample.

For experiments designed to test the effect of L-NAME on EC proliferation under varying WSS, the percentage of EC proliferation (the variable \(Y\)) was modeled as a function of WSS in presence or absence of the drug (L-NAME takes the value of 1 or 0):

\[
Y = \beta_0 + \beta_1(WSS) + \beta_2(L-NAME) + \beta_3(L-NAME) + \varepsilon
\]

where coefficient \(\beta_2\) represents the effect of L-NAME, while coefficient \(\beta_3\) quantifies the interaction between WSS and L-NAME, and \(\varepsilon\) is the random error of the sample. For each regression analysis, more than 120 data points were included, from three separate experiments, with ~40 WSS values in each experiment. Student’s t-test was used to compare results of parallel chamber experiments with static controls. The criterion for statistical significance was set at \(\alpha = 0.05\).

**RESULTS**

**Flow dynamics in the tapered chamber.** The tapered chamber was designed to study the effect of various WSS conditions simultaneously in a single experiment. CFD simulations confirmed that the flow was laminar and accelerating along the distance in the test section as shown in Fig. 2A. The Reynolds number was 465 in the entrance of the tapered section and increased to 570 at the exit. The pressure in the entrance of the
The tapered section was 100 mmHg and dropped to 96 mmHg at the exit. WSS increased monotonically along the flow direction from 7 dyne/cm² at the beginning of the taper to 154 dyne/cm² at the end as shown in Fig. 2B.

**Morphological responses of ECs to high WSS.** In the tapered chamber, ECs were subjected to WSS for 24 or 48 h. Light microscopic examination revealed a confluent monolayer for all WSS levels. In contrast to reports from studies using subconfluent monolayers (17, 31), density measurements revealed that there was no significant cell loss with increasing WSS (mixed regression analysis, \( \beta_1 = -0.15; P = 0.48 \); Fig. 3), indicating that high WSS did not have obvious deleterious effects. Furthermore, there was minimal mechanical detachment of cells at high WSS, since fewer than 0.5% of the cells could be detected in the recirculating media when cells were exposed to a uniform WSS of 85 dyne/cm² using the parallel chamber. Mean cell viability among the attached cells, assessed using Trypan blue, was more than 98% under all tested shear conditions.

A further indication that the cells were alive and functional, even at high WSSs, was the fact that they elongated and oriented with the flow direction. The time course of this rearrangement varied with WSS. Cells exposed to WSS up to 40 dyne/cm² were all aligned parallel to flow at 24 h. In contrast, cells exposed to 40–60 dyne/cm² displayed no preferred orientation at 24 h, and cells exposed to >60 dyne/cm² became oriented perpendicular to flow at 24 h (Fig. 4). However, by 48 h, cells at all tested WSS conditions were aligned parallel to flow. Such transient perpendicular orientation of ECs in high flow has been previously reported (30).

**High WSS stimulates EC proliferation.** Because all cells were aligned parallel to the flow (as ECs are in vivo) at 48 h, this time was chosen for studying EC proliferation. In agreement with previous studies (2), incorporation of BrdU revealed that WSS between 15 and 25 dyne/cm² inhibited proliferation compared with the proliferation of cells under static conditions (Fig. 5). In contrast, WSS above 30 dyne/cm² enhanced proliferation, revealing a bimodal effect of WSS on EC proliferation.

However, compared with the proliferation at the accepted physiological baseline of 15–20 dyne/cm², high WSS (≥20 dyne/cm²) overall stimulated proliferation. Specifically, the percentage of proliferating ECs rose rapidly from 0.9% to 6% as WSS increased from 15 to 50 dyne/cm² (Fig. 5). Mixed regression analysis showed that WSS significantly stimulated proliferation (\( \beta_1 = 0.06; P < 0.0001 \)), with the effect being most dramatic between 15 and 30 dyne/cm², where the slope of the proliferation increase was steepest (\( \beta_1 = 0.1949; P < 0.0001 \)). Further increase in WSS from 40 to 100 dyne/cm² further stimulated proliferation, albeit to a lesser extent (\( \beta_1 = 0.0214; P < 0.0005 \); Fig. 5).

To confirm that high WSS stimulated cell proliferation, we also subjected ECs to flow in a commercial chamber with parallel sides to provide a uniform WSS field to the EC monolayer. At 24 h, cells exposed to 85 dyne/cm² were aligned perpendicular to the flow as they did at the high WSS region of the tapered chamber. Comparison of BrdU incorporation in cells exposed to a uniform WSS of 85 dyne/cm² showed higher proliferation rates compared with static controls (Fig. 6). Student’s t-test showed that the difference was significant (\( t = 9.92, 2 \) degrees of freedom; \( P = 0.01 \)).

**High WSS reduces EC apoptosis.** High WSS not only stimulated proliferation, but also suppressed cell death. Staining for apoptotic cells using the TUNEL assay revealed that cell apoptosis decreased with increasing WSS. Mixed regression analysis showed that the negative effect of WSS on apoptosis was significant (\( \beta_1 = -0.023; P < 0.0001 \); Fig. 7).

**NO production is necessary for high WSS stimulation of EC proliferation.** Bao et al. (4) have reported that physiological WSS conditions induce ECs to produce NO at a level that inhibits proliferation, whereas flow characterized by high temporal gradients in WSS causes excessive endothelial NO production that appears to stimulate proliferation. Because NO production can apparently have different effects on EC proliferation depending on the flow conditions, we investigated if NO mediates the stimulation of ECs by high WSS. To this end, we inhibited NOS activity with L-NAME, whereas ECs were subjected to flow for 24 h, an exposure that stimulated cell proliferation the same way as at 48 h (Fig. 8A). We confirmed that, over 24 h, 1 mM L-NAME did not affect EC proliferation under static conditions, as others have reported (11). We also...
observed that L-NAME-treated cells still oriented to the flow direction similar to cells in the absence of inhibitor, and the EC monolayer remained continuous without disruptions and with uniform cell density across the glass slide after 24 h under flow in the presence of the inhibitor (data not shown). Mixed regression analysis, using Eq. 2, indicates that NO inhibition did not significantly reduce EC proliferation compared with the nontreated group for WSS $<30$ dyn/cm$^2$ ($\beta_2 = -2.282; P = 0.057$), which is consistent with a previous study (11). However, for WSS $>30$ dyn/cm$^2$, the EC proliferation was significantly reduced in the L-NAME-treated cells compared with the nontreated cells ($\beta_2 = -4.56; P = 0.019$). Furthermore, the term expressing interaction between the effects of L-NAME and WSS on EC proliferation was also significant ($\beta_3 = -0.072; P < 0.001$). In other words, the slope of EC proliferation vs. WSS was significantly altered by the presence of L-NAME. This means that L-NAME influenced the EC response to WSS (Fig. 8A), further implicating NO as a critical mediator in the stimulation of EC proliferation by WSS.

Treatment of ECs in vitro with a WSS of 15–20 dyn/cm$^2$ has been shown to stimulate NO production by increasing eNOS expression (22, 28). Therefore, we examined eNOS protein expression by ECs in the tapered chamber using immunofluorescence staining. Staining for eNOS protein at 24 h showed an increase in eNOS expression with increasing WSS, reaching a twofold increase at $\sim40$ dyn/cm$^2$ compared with 15 dyn/cm$^2$ (Fig. 8B). Mixed regression analysis, using Eq. 1, showed that with increasing WSS there was a significant increase in eNOS expression compared with static control baseline. Each point represents the average of 3 experiments ± SE. WSS significantly stimulated proliferation (mixed regression analysis, $\beta_1 = 0.06; P < 0.0001$).

Fig. 4. EC alignment at various WSS conditions, using the tapered chamber, at 24 and 48 h. Cells oriented to the direction of the flow with a time course that varied with WSS.

Fig. 5. Percentage of proliferating cells [bromodeoxyuridine (BrdU) positive] increased with increasing WSS at 48 h. During the flow experiments, time-matched cell cultures at no-flow conditions provided the "static control baseline" of EC proliferation. The 2 shaded regions emphasize the opposite proliferative EC behaviors at 15–25 dyn/cm$^2$ vs. higher WSSs ($<30$ dyn/cm$^2$) compared with the static control baseline. Each point represents the average of 3 experiments ± SE. WSS significantly stimulated proliferation (mixed regression analysis, $\beta_1 = 0.06; P < 0.0001$).

Fig. 6. Parallel chamber results. The proliferation at 85 dyn/cm$^2$ was significantly higher compared with that under static conditions at 24 h. Bars represent the average of 3 experiments ± SE. *Student’s $t$-test, $t = 9.92$, 2 degrees of freedom; $P = 0.01$. 

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expression ($\beta_1 = 4.8 \times 10^{-3}; P < 0.005$). The dependence of eNOS expression on WSS had a very similar trend to that of EC proliferation at 48 h: eNOS increased between 15 and 40 dyn/cm$^2$, and above 40 dyn/cm$^2$ it reached a plateau (Fig. 8B). Compared with static control, eNOS intensity was upregulated under all tested shear stress conditions.

DISCUSSION

In this study, by isolating the EC response to hemodynamics, we showed that high WSS (above the level of 15–20 dyn/cm$^2$ that is commonly accepted as the physiological baseline) stimulates EC proliferation. Whereas previous in vivo studies have demonstrated that a chronic increase in flow through an arterial segment is followed by increased EC proliferation during normal adaptive arterial expansion (25), in vivo systems involve many confounding factors such as tensile stress (which stretches the vessel wall), EC-smooth muscle cell interactions, and cell-matrix interactions, which cannot be easily separated. The present study demonstrates not only that high WSS can stimulate EC proliferation, but also that increased EC proliferation is a direct response of the ECs themselves.

To our knowledge, there is only one previous study that investigated the EC growth response to WSS values significantly higher than 30 dyn/cm$^2$ in vitro (17). It was found that when subconfluent ECs were exposed to flow for 24 or 48 h, they had decreased cell densities with increasing WSS. However, the reduction in cell density at higher WSS was later found to be due to mechanical detachment and not to an inability of the cells to proliferate (31). Thus subconfluent ECs display different behavior from a confluent monolayer, presumably due to the lack of cell-cell contact. Our results clearly demonstrate a bimodal response of confluent ECs to WSS. Whereas WSS up to 30 dyn/cm$^2$ inhibits proliferation compared with static conditions, as shown in Fig. 4 and in previous studies (2), WSS above 30 dyn/cm$^2$ stimulates proliferation. The appropriate reference point for considering the role of WSS in the regulation of EC proliferation should not be the static culture, as used in most previous in vitro studies, but should be ECs under 15–20 dyn/cm$^2$, because this is the in vivo baseline condition. By considering the EC proliferation at 15–20 dyn/cm$^2$ as the baseline behavior, it is clear that WSS can have a stimulatory effect on proliferation in high WSS environments, in addition to the inhibitory effect previously reported in low WSS environments.

The proliferation of cells under high WSS is not the result of damage or cell loss in the high-shear region and subsequent replacement. Not only was there an absence of detectable cells in the recirculating media, but there was also reduced apoptosis in high WSS regions (Fig. 7) suggesting that high WSS promotes EC survival. Thus the increased cell proliferation at high WSS is a direct response to WSS and not an indirect response to some kind of cell loss.

Our study shows that NO is required for the WSS-stimulated proliferation, since the NOS inhibitor, L-NAME, drastically reduced the shear-induced proliferation of ECs (Fig. 8A). NO is known to be necessary for outward remodeling of blood vessels in response to chronic high flow (27) and specifically to be involved in activation of matrix metalloproteinases for extracellular matrix remodeling (26). NO has also been shown to activate the mitogen-associated kinase pERK1/2 in ECs (4). Our results further show that NO is necessary for the direct stimulation of EC proliferation by high WSS.

Our study indicates that high WSS stimulates NO production by upregulating eNOS. The eNOS gene is known to have a shear stress response element, and we show that eNOS expression increased in parallel with cell proliferation in response to elevated WSS. Shear-induced increase in eNOS mRNA has
been shown to require calcium influx (19), suggesting that a possible mechanism by which ECs sense high WSS is through stretch-activated calcium channels (SACs) on the luminal surface of the endothelium (5). WSS exerts a tangential force on the endothelium that could stretch the surface, and the mechanosensitivity and density of SACs on ECs has been shown to increase when WSS increases from 5 to 15 dyn/cm² (5), potentially amplifying this response. Another possible mechanotransducer for WSS is the VEGF receptor 2 (VEGFR-2), which can be activated by flow without the participation of its ligand (6), although the molecular mechanism for this is unknown. Like SACs, the density and activity of VEGFR-2 also increase with increasing flow (24).

Although endothelial damage has been associated with regions of high WSS during aneurysm initiation in vivo (8, 9), our experiments show that high WSS alone did not compromise the integrity of an EC monolayer, but, rather, it stimulated EC proliferation and inhibited apoptosis. This behavior may be beneficial in regions of sustained high WSS, where it is reasonable to expect more mechanical damage and cell turnover. Indeed, it has been shown that mitotic ECs along the aorta are found almost exclusively at the base of arterial branches, indicating that endothelial proliferation is particularly high around bifurcations (32). It is noteworthy that hypertension, a well-recognized risk factor of atherosclerosis development, is believed to reduce the bioavailability of NO (12, 23), and we find that NO is necessary for the WSS-stimulated proliferation. Reduced NO bioavailability could disrupt the protective responses in ECs elicited by high WSS, rendering regions such as apices of bifurcations and sharp curvatures more susceptible to mechanical damage when subjected to sustained hemodynamic stress.

In conclusion, very high WSS, such as occurs with compensatory flow increase in collateral arteries or at apices of bifurcations and outer sides of curvatures, can directly stimulate EC proliferation, and this stimulation is dependent on a NO signaling mechanism. Stimulation of EC proliferation by high WSS is likely to play an important role in maintaining continuity of the endothelium in regions where the vessel wall is subjected to high levels of hemodynamic stress. Furthermore, the regulation of this behavior by NO offers a direct connection to physiological risk factors that are known to potentiate atherosclerosis formation in these hemodynamic environments.

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DISCLOSURES

John Kolega is a consultant for Boston Scientific (for a project not directly related to the topic of this article).

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dysfunction in hypertension is independent from the etiology and from vascular structure. *Hypertension* 31: 335–341, 1998.


