Pulsatile Flow–Induced Angiogenesis
Role of G_{i} Subunits

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Objective—Angiogenesis plays a key role in the growth and function of normal and pathological tissues. We investigated the effect of pulsatile flow on endothelial cell (EC) in vitro angiogenic activity. Methods and Results—Bovine aortic ECs were exposed to “static” or “flow” (1.2 to 67.0 mL/min, shear stress 1.4 to 19.2 dyne/cm\(^2\)) conditions for 2 to 24 hours. After exposure, angiogenesis was measured as tubule formation on Matrigel, and EC migration was assessed by filter migration assay. Pulsatile flow increased angiogenesis and EC migration in a temporal and force-dependent manner, with a maximal effect at 16 hours (13.2 dyne/cm\(^2\)). Pertussis toxin completely inhibited the effect of pulsatile flow on angiogenesis and migration. Transfection of ECs with inhibitory mutants of the \(\alpha\) subunit of G_{1} or G_{3}, but not G_{2}, inhibited the flow-induced angiogenic response by 61\% and 32\%, respectively, whereas transfection with constitutively activated mutants of the \(\alpha\) subunit of G_{1} or G_{3}, but not G_{2}, increased the flow-induced response by 202\% and 70\%, respectively. In contrast, inhibition of G_{i} by the carboxy terminal fragment of \(\beta\)-adrenergic receptor kinase overexpression increased the flow-induced response by 82\%.

Conclusions—These results suggest that pulsatile flow stimulates angiogenesis and that this effect is mediated by activation of G_{1} or G_{3}, but not G_{i} subunits. (Arterioscler Thromb Vasc Biol. 2002;22:1610-1616.)

Key Words: shear stress ■ angiogenesis ■ G proteins ■ endothelial cells ■ migration

Endothelial cells (ECs) are present in the vasculature as a monolayer and constitute the interface between the bloodstream and vessel wall. Because of their strategic location, ECs are constantly subjected to a variety of mechanical forces resulting from pulsatile blood flow. These hemodynamic forces, including shear stress and pressure, profoundly affect EC biology and thus play an important role in vasoregulation and vascular remodeling and in the pathogenesis of atherosclerosis. Fluid shear stress, considered the principal stimulus for ECs, can influence a variety of EC functions, including the production of vasoactive mediators and the expression of cell adhesion molecules, and is the primary driving force for control of blood vessel architecture.

Angiogenesis is the formation of new capillaries from the preexisting vasculature by migration and proliferation of ECs, and it plays a fundamental role in the growth, survival, and function of normal and pathological tissues. The process of angiogenesis requires loosening of intercellular junctions and degradation of the pericellular matrix by ECs, migration of the EC toward the angiogenic stimulus, sprout formation, formation of a lumen, and the joining of sprouts to form a capillary bed. Angiogenesis may be beneficial in some clinical circumstances, such as in tissue damage after reperfusion of ischemic tissue or cardiac failure, but maladaptive in other situations, such as cancer and intraplaque formation. An association between blood flow/shear stress and angiogenesis is supported in the literature. High blood flow occurs concomitantly with capillary growth, or angiogenesis, in physiological conditions such as exercise or exposure to high altitude and is also a feature of tumors, in which its restriction has been used successfully to cause their regression. It has been demonstrated that physiological shear stress enhances wound closure in cultured human umbilical vein and coronary artery ECs via the action of EC spreading and migration, and a correlation has been reported between blood flow and angiogenesis in a variety of animal models. Vasodilation precedes capillary growth during wound healing, and it is an accompanying sign of other inflammatory diseases linked with angiogenesis, such as arthritis or psoriasis. However, the underlying mechanism remains unclear.

Heterotrimeric G proteins, composed of \(\alpha\), \(\beta\), and \(\gamma\) subunits, function as transducers of information across the cell membrane by coupling diverse receptors to effectors;
thus, they play a central role in signal transduction and cell biology.12 Recent evidence suggests that Gi1, Gi2, and Gi3, which are pertussis toxin (PTX) sensitive, and Gq, which is PTX insensitive, may play a role in agonist-induced and shear stress–induced responses, including the release of vasoactive substances, such as NO and prostacyclin (PGI2), from ECs.13–15 Stimulation of these G proteins triggers a number of signal transduction cascades, including activation of K+ channels, phospholipase A2, phospholipase C, and adenylyl cyclase.16–18 Whereas G-protein–coupled receptors (GPCRs) have been implicated in the control of a number of angiogenic signals,19 the role of specific G-protein subunits in transducing the effect of shear stress on angiogenesis has not been established.

Using an in vitro perfused cell culture system, we investigated the direct effect of shear stress on EC angiogenic activity and determined the role of PTX-sensitive G proteins in transducing the signal. We demonstrate that pulsatile flow induces EC migration and angiogenesis in a flow- and time-dependent manner via a Gi1/Gi3-dependent, Gi2-independent, signal transduction pathway.

Methods

An expanded Methods section is available online at http://atvb.ahajournals.org.

Vascular ECs

Bovine aortic ECs (BAECs), repository No. AG07680B, were obtained from the National Institute on Aging Cell Culture Repository, Coriell Institute for Medical Research. These cells tested positively for the EC-specific von Willebrand factor and tested negatively for α-smooth muscle actin. BAECs were seeded into polyethylene capillaries (capillary length 13 cm, internal diameter 330 μm, wall thickness 50 μm, pore size 0.3 μm/μL, extracapillary surface area 123 cm², and luminal surface area 108 cm²) through which medium from a reservoir is pumped in a pulsatile fashion, at a chosen flow rate, via silicone capillaries (capillary length 13 cm, wall thickness 50 μm, pore size 0.3 μm/μL). The perfused transcapillary cultures were designated as

Flow-Induced Angiogenesis and G Proteins

perfused transcapillary cultures. Cultures of BAECs were established as described in detail previously.20–22 The CELLMAX QUAD Artificial Capillary Cell Culture System (Spectrum Laboratories) was used. This apparatus consisted of an enclosed bundle of 150 permeable Prorectin–F–coated (Deepwater Chemicals Inc) polyethylene capillaries (capillary length 13 cm, internal diameter 330 μm/μL, wall thickness 50 μm/μL, pore size 0.3 μm/μL, extracapillary surface area 123 cm², and luminal surface area 108 cm²) through which medium from a reservoir is pumped in a pulsatile fashion, at a chosen flow rate, via silicone rubber tubing. By alteration of the flow rate, a shear stress range of 1.4 to 19.8 dynes/cm² can be achieved in this system.

Seedng of BAEC Experimental Protocol

BAECs in RPMI 1640 supplemented with 1% FBS were seeded into the lumen spaces of the capillary bundle at such a density as to ensure confluence, as detailed previously.18,20–22 The experimental protocol followed is shown diagrammatically (please see online Figure I, which can be accessed at http://atvb.ahajournals.org). Perfused transcapillary cultures were designated as “flow,” and culture flasks, precoated with fibronectin, of confluent BAEC were designated as “static.” The angiogenic response of BAECs cultured under static conditions on fibronectin-coated flasks was not significantly different from that of BAEC cultured on Prorectin–F–coated flasks or on Prorectin–F–coated cartridges. After the 12-hour stabi-

lization period, the flow group was exposed to a single-step increase in pulsatile flow up to the designated flow rate/shear stress and maintained at that rate for the indicated period of time (2 to 24 hours). For a flow rate of 43.6 mL/min, shear stress was 13.2 dynes/cm², the intraluminal pulse pressure was 107/7 mm Hg (amplitude 100 mm Hg), and the frequency was 4 Hz. Where indicated, cultures were treated with PTX (100 ng/mL). At the end of the experimental period, BAECs were harvested from the carriages and used in the migration and angiogenesis assays, or membranes/lysates were prepared for Western blot analysis.

Transwell Filter Migration Assay

Fibronectin-coated Transwell filters (12-μm pore size, Costar) were used for migration assays. BAECs harvested from static experiments or the transcapillary cultures were seeded at a density of 5×10⁴ cells per filter. For migration studies, the cells were allowed to migrate for 10 hours with conditioned BAEC media in the upper and lower chambers. In this way, random migration or chemokinesis was measured because there was no concentration gradient between the upper and lower chambers. After the fixation and staining procedures, the number of cells that migrated through the filter pore was manually counted per high power field (hpf) by using a microscope (Nikon Diaphot). Data are reported as the number of BAECs counted per 10 hpf and are expressed as a percentage of control, where control indicates BAECs exposed to static conditions unless otherwise stated.

Angiogenesis Assay

The wells of 96-well tissue culture plates were coated with Matrigel basement membrane matrix (100 μL per well, Becton Dickinson), which was allowed to solidify at 37°C for 30 minutes, according to the manufacturers instructions, before plating the cells. The cell suspension, conditioned medium, containing 3×10⁴ cells was then plated at 125 μL per well onto the surface of the Matrigel and incubated at 37°C. Sixteen hours later, the cells were photographed with the use of a CCD digital camera (Spot RT, Diagnostics Instruments, Inc) at ×4 magnification. Tube formation was quantified by measuring the length of the network of connected cells in each well with the use of Scion Image for Windows (Scion Corp).

Preparation of Cell Lysates

Harvested BAECs were pelleted by low-speed centrifugation. The cell pellet was placed in ice-cold lysis buffer and subjected to ultrasonication with a sonic dismembrator (Fisher Scientific). Samples were divided into aliquots and stored at −80°C before use for Western blot analysis. Protein concentration was measured by the method of Bradford, with BSA used as a standard.

Western Blotting

Cell lysates (10 to 20 μg per lane) were analyzed for G-protein expression by Western blot analysis. Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Hybond-C, Amersham Pharmacia Biotech) by using a Mini Trans-Blot Cell (Bio-Rad) at 80 V for 1 hour. Anti-Gi1, anti-Gi2, and anti-Gi3 antibodies were obtained from Upstate Biotechnology, and the controls Gi1, Gi2, and Gi3 were obtained from Santa Cruz Biotechnology, Inc.

Transfections

Plasmid DNA was transfected into cells by use of LipoFectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions; green fluorescent protein (GFP) was used as an internal control. Briefly, after trypsination, BAECs were cotransfected with the active or inactive mutants of Gi and GFP in suspension for 10 minutes. Cells were then reseeded into tissue culture flasks for 24 hours, after which they were subjected to trypsination and seeded onto fibronectin-coated dishes or in the transcapillary culture systems, intraluminally, as described above. Because cotransfection with 2 independent vectors results in the internalization of both plasmids by the same cell,23 cells that were transfected with the Gi mutants fluoresced
because of the presence of GFP. Cells were also transfected with the carboxy-terminal fragment of β-adrenergic receptor kinase (ßark-ct). The cDNA clones for G_i-1-G202T, G_i-1-Q204L, G_i-2-G203T, G_i-2Q205L, G_i-3-G202T, and G_i-3-Q204L were provided by the Guthrie cDNA Resource Center (Sayre, Pa). Plasmid cDNA3.1 was obtained from Invitrogen.

Statistical Analysis

Results are expressed as mean±SEM, and n indicates the number of individual perfused transcapillary cultures from which cells were harvested. Experimental points were performed in triplicate, with a minimum of 3 independent experiments. Experimental and control cells in migration and angiogenesis assays were compared by use of unpaired 2-tailed Student t tests. When >2 groups were present, ANOVA (factorial design) was used (GraphPad Prism). A value of P<0.05 was considered significant.

Results

Effect of Pulsatile Flow on EC Migration and Angiogenesis

The temporal and force-dependent effects of pulsatile flow on EC migration and angiogenesis were first determined. There was a significant flow-dependent increase in migration (assessed by Transwell assay) and angiogenesis (assessed by tubule formation on Matrigel) after exposure of the cells to flow rates of 4.6 to 65.3 mL/min (corresponding to shear stresses of 1.4 to 19.8 dyne/cm², respectively) over a period of 16 hours compared with static conditions (Figure 1). Treatment of static BAECs with conditioned media (ie, media from BAECs exposed to 16 hours of flow) during the Matrigel assay had no significant effect on tubule formation compared with the effect on BAECs treated with control/nonconditioned media (data not shown). The flow-induced BAEC response was time dependent, with a maximal effect observed for migration and angiogenesis at 16 hours (Figure 2). For all subsequent experiments, BAECs were exposed for 16 hours to a flow rate of 43.6 mL/min, corresponding to a shear stress of 13.2 dyne/cm².

Effect of PTX on Flow-Induced EC Migration and Angiogenesis

The effect of pharmacological inhibition of G_i activity was determined by exposing BAECs to pulsatile flow (13.2 dyne/cm², 16 hours) in the presence or absence of PTX before their migration and angiogenesis were assessed. Migration and angiogenic activity of the static cells were not significantly affected by treatment with PTX (100 ng/mL). In contrast, PTX treatment completely inhibited the pulsatile
flow–induced EC migratory (Figure 3a) and angiogenesis (Figure 3b) responses.

**Effect of G\_i\_1 Transfection on Flow-Induced Angiogenesis**

To allow a more definitive evaluation of the role of G\_i\_1-mediated events in pulsatile flow–induced angiogenesis, BAECs were transfected with constructs for the appropriate active and inactive mutants of G\_i\_1. Although difficulties in the transfection of some cell types exist, we have successfully transfected BAECs with high efficiency (50% to 60%), as demonstrated by cotransfection with a plasmid expressing GFP (data not shown). As assessed by Western blot, transfection with the mutant constructs selectively enhanced the expression of the targeted G\_i\_1 subunit in the absence of any effect on the other G\_i\_ subunits (please see online Figure II, which can be accessed at http://atvb.ahajournals.org). Transfection of the mutant constructs had no significant effect on the angiogenic activity of BAECs exposed to static conditions (data not shown).

Transfection with the inhibitory mutant of the α subunit of G\_1 (G\_i\_1-G202T) resulted in a 61±2% decrease in the pulsatile flow–induced angiogenic response, whereas transfection with a constitutively activated mutant of the α subunit of G\_1 (G\_i\_1-Q204L) led to a 202±23% increase in the response compared with the response of cells transfected with the empty vector, pcDNA3.1+ (Figure 4a).

**Effect of G\_i\_2 Transfection on Flow-Induced Angiogenesis**

When BAECs were transfected with the inhibitory (G\_i\_2-G203T) or constitutively activated (G\_i\_2-Q205L) mutant of the α subunit of G\_2 before exposure to pulsatile flow, there was no change in the angiogenic response of these cells compared with the response of cells transfected with the empty vector (Figure 4b).

**Effect of G\_i\_3 Transfection on Flow-Induced Angiogenesis**

Transfection with the inhibitory mutant of the α subunit of G\_3 (G\_i\_3-G202T) resulted in a 32±6% decrease in the pulsatile flow–induced angiogenic response, whereas transfection with a constitutively activated mutant of the α subunit of G\_3 (G\_i\_3-Q204L) led to a 70±4% increase in the response compared with the response of cells transfected with the empty vector (Figure 4c). Cotransfection of G\_i\_1/G\_i\_3-G202T resulted in a 73±6% inhibition of the flow-induced angiogenic response (Figure 4d).

**Effect of βark-ct Transfection on Pulsatile Flow-Induced Angiogenesis**

The pulsatile flow–induced angiogenic response was significantly increased (by 82±8%) in BAECs transfected with βark-ct, a 194-amino-acid peptide responsible for binding and inhibiting the G\_β\_γ subunit (Figure 4e).

**Discussion**

Angiogenesis is associated with a large number of pathological situations, such as tumor growth and arthritis, and is recognized as an important process required for the progres-
sion of atherosclerosis. However, it is beneficial in other circumstances, such as wound healing and ischemia. The main findings of the present study are that shear stress stimulates ECs to increase their angiogenic activity by a mechanism transduced via the G-protein isoforms Gi1 and Gi2. In particular, the shear stress–induced angiogenic response was inhibited by PTX, which ADP-ribosylates and inactivates Gi1, Gi2, and Gi3. Precisely which PTX-sensitive Gi subunit(s) was involved in transducing the response was determined by overexpressing inhibitory or constitutively activated mutants of Gi1, Gi2, and Gi3 in ECs before assessing their response to flow. The contribution of Gβγ was investigated after overexpression of the inhibitory mutant, βark-ct. These studies support a physiologically important role for shear stress in modulating vascular remodeling during angiogenesis.

Although angiogenesis can be induced by diverse growth factors, increased or decreased blood flow and/or blood pressure is linked with growth of the vessels or their regression under many circumstances, and previous studies have pointed to a relationship between blood flow, its associated hemodynamic forces, and new blood vessel formation. Indeed, Nasu et al. concluded from their study that tumor angiogenesis depended more on local hemodynamics than on vascular growth factors. In these blood flow–dependent phenomena, wall shear stress acting at the vascular EC surface is believed to play a key role. Numerous in vivo and in vitro studies have demonstrated that shear stress can modulate the morphology and many functions of ECs. More recently, a cis-acting shear stress–responsive element was identified in the promoters of ECs that respond to shear stress, suggesting a common mechanism linking biomechanical forces to gene expression. DNA microarray analysis of gene expression in ECs revealed that genes involved in survival and angiogenesis (Tie2 and Flk-1) were upregulated by shear stress. However, details of the process by which shear stress–mediated changes in EC functions lead to vascular remodeling and angiogenesis are not entirely clear. The present study demonstrates a direct modulatory effect of shear stress on EC migration, which is essential for neovascularization, and on EC angiogenic activity. Our migration data are in agreement with data from Albuquerque et al and

Figure 4. Effect of Gβγ subunit/βark-ct transfection on pulsatile flow–induced BAEC angiogenesis. BAECs were transfected as follows: panel a, Gα1-G202T (inhibitory mutant of the α subunit of G1) or Gα1-Q204L (constitutively activated mutant of the α subunit of G1); panel b, Gα2-G203T (inhibitory mutant of the α subunit of G2) or Gα2-Q205L (constitutively activated mutant of the α subunit of G2); panel c, Gα3-G202T (inhibitory mutant of the α subunit of G3) or Gα3-Q204L (constitutively activated mutant of the α subunit of G3); panel d, Gα1-G202T and Gα3-Q204T (cotransfection); and panel e, βark-ct (inactive mutant of the βγ subunit). Transfected cells were exposed to static or flow (13.2 dyne/cm², 16 hours) conditions before their angiogenic activities were assessed. Angiogenesis is expressed as tubule formation on Matrigel as percent control, ie, flow–induced angiogenesis of cells transfected with empty vector (pcDNA3.1+). Data are mean±SEM (n=4 to 7). *P<0.05 vs control.
Urbich et al.\textsuperscript{27} who demonstrated that shear stress enhanced EC migration, as assessed by scratch wound assay. The former group used a parallel-plate flow chamber, and the latter group used a cone-and-plate apparatus to expose ECs to steady laminar shear stress. Shear stress–induced migration was independent of any effect on cell proliferation and dependent on the fibronectin receptor $\alpha_5\beta_1$.\textsuperscript{27} Unlike the present study, however, the effect of shear stress on angiogenesis and the role of specific G-protein subunits in mediating the response were not addressed by these researchers.

Our data suggest that exposure of ECs to flow for at least 2 hours results in an altered phenotype, which is retained through subsequent harvesting and replating. However, the possibility that pulsatile flow exposure results in the detachment of some cells, leaving a “sub-selected” more angiogenic population attached, cannot be completely discounted. This is unlikely, however, because we have previously determined by scanning electron microscopy that an intact EC monolayer is present after flow exposure.\textsuperscript{20} In addition, previous studies have demonstrated that ECs become more adherent after shearing.\textsuperscript{28}

A role for GPCRs (PTX sensitive and insensitive) in transducing shear stress–induced responses in ECs has been previously recognized.\textsuperscript{13–15} Possible mechanisms of activation of these GPCRs by hemodynamic forces include indirect activation after stimulated release of a substance by the ECs or by a direct mechanical effect.\textsuperscript{1,2} The fact that conditioned media from ECs exposed to pulsatile flow had no effect on static EC angiogenesis points to a role for G proteins acting as primary mechanosensors. Furthermore, in a study by Gudi et al.\textsuperscript{29} G proteins reconstituted in liposomes, in the absence of protein receptors, showed an increase in activity in response to shear stress, supporting a role for G proteins of protein receptors, showed an increase in activity in response to shear stress, supporting a role for G proteins acting as primary mechanosensors in ECs. Although we demonstrate a role for $G_{\alpha}$ subunits in transducing shear stress responses in ECs, not all $G_{\alpha}$ subunits were equally effective. Our data suggest a more important role for $G_{\alpha}1$ and $G_{\alpha}3$ than for $G_{\alpha}2$ in mediating the flow-induced angiogenesis response. Interestingly, of the 3 different isoforms, $G_{\alpha}1$ and $G_{\alpha}3$ are the most closely related. Comparison of the amino acid sequences of the 3 isoforms from human or rat revealed that $G_{\alpha}1$ and $G_{\alpha}3$ are identical at 94% of their amino acids, whereas either $G_{\alpha}1$ or $G_{\alpha}2$ was 86% to 88% identical to $G_{\alpha}3$. Such a comparison may facilitate determination of the domain by which $G_{\alpha}$ subunits either sense shear stress or how they interact with their partner subunits.

It is noteworthy that transfection of $G_{\alpha}$ mutant constructs (dominant negative or constitutively active) had no significant effect on the angiogenic activity of BAECs exposed to static conditions. These data support the concept of distinct signal transduction pathways mediating “basal” angiogenesis and the shear stress–stimulated angiogenic response.

Much of the initial focus after the discovery of heterotrimeric G proteins was directed at the G-protein $\alpha$ subunit. However, it is now recognized that $\beta\gamma$ subunits also play an important role in cellular signaling via multiple effector molecules that include adenyl cyclase, phosphatidyl inositol-3-kinase, and mitogen-activated protein kinase.\textsuperscript{30,31} $\beta\gamma$ also inactivates the activity of Go by reassociation with the subunit, whereby it enhances $\alpha$-subunit GTPase activity.\textsuperscript{30}

In the present study, we demonstrate that inhibiting $\beta\gamma$ by overexpression of $\beta\gamma$-ark-ct resulted in an enhanced flow-induced angiogenic response. One possible mechanism to explain this response is that under flow conditions, the $\beta\gamma$ subunit has an inhibitory effect on the angiogenic response and that by blocking $\beta\gamma$ activity with $\beta\gamma$-ark-ct, this inhibitory effect is attenuated, leading to an augmented response. However, this hypothesis is unlikely, inasmuch as it has been shown by Bicknell and Valle\textsuperscript{e} that the activation in ECs of phospholipase C, an effector molecule of the $\beta\gamma$ subunit, by angiogenin, induces vascularization in classic angiogenesis assays. It is more probable that the inhibitory effect of $\beta\gamma$-ark-ct on the $\beta\gamma$ subunit prevents its reassociation with the $G_{\alpha}$ subunit(s), leading to prolonged activation of these subunits and thus to an increase in the angiogenic response.

In summary, our findings suggest that shear stress is a physiologically relevant stimulus for EC migration and angiogenesis and highlight the role of specific G-protein subunits in mediating this response.

**Acknowledgments**

This work was supported in part by a grant from the National Institutes of Health (AA12610 to Dr Redmond). Dr Cullen was the recipient of a Postdoctoral Fellowship Award from the American Heart Association, New York State Affiliate.

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Arterioscler Thromb Vasc Biol. 2002;22:1610-1616; originally published online August 22, 2002;
doi: 10.1161/01.ATV.0000034470.37007.58
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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