Cyclic Strain Regulates the Notch/CBF-1 Signaling Pathway in Endothelial Cells
Role in Angiogenic Activity

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Objective—The purpose of this study was to determine the effect of cyclic strain on Notch signaling in endothelial cells.

Methods and Results—Exposure of human endothelial cells (ECs) to cyclic strain (10%) resulted in temporal upregulation of Notch receptors (1 and 4) at the mRNA and protein level. Cyclic strain significantly increased EC network formation on Matrigel (an index of angiogenesis); network AU = 775 ± 127 versus 3928 ± 400 for static and strained ECs, respectively. In addition, Angiopoietin 1 (Ang1), Tie1, and Tie2 expression were increased and knockdown of Ang1/Tie1,2 by siRNAs decreased cyclic strain-induced network formation. Knockdown of Notch 1 and 4 by siRNA, or inhibition of Notch mediated CBF-1/RBP-Jk regulated gene expression by RPMS-1, caused a significant decrease in cyclic strain-induced network formation and in Tie1 and Tie2 mRNA expression. Notch 1 or Notch 4 siRNA, but not RPMS-1, inhibited cyclic strain-induced Ang1. Constitutive overexpression of Notch IC resulted in increased network formation, and Ang1 and Tie2 mRNA expression, under both static and strain conditions.

Conclusions—These data suggest that cyclic strain-stimulated EC angiogenesis is mediated in part through a Notch-dependent, Ang1/Tie2 signaling pathway. This pathway may represent a novel therapeutic target for disease states in which hemodynamic force-induced angiogenesis occurs. (Arterioscler Thromb Vasc Biol. 2007;27:1289-1296.)

Key Words: mechanical forces ■ Notch ■ endothelial cells ■ angiogenesis ■ angiopoietin

Because of their strategic location, at the interface of the bloodstream and the vessel wall, endothelial cells are constantly subjected to a variety of mechanical forces resulting from pulsatile blood flow. These hemodynamic forces, including cyclic strain and shear stress, profoundly affect endothelial biology and play an important role in vasoregulation, vascular remodeling, and the pathogenesis of atherosclerosis.1-2 More recently a relationship between hemodynamic forces and angiogenesis, ie, the formation of new capillaries by migration and proliferation of endothelial cells, has been established. High blood flow occurs concomitantly with angiogenesis in physiological conditions such as exercise or exposure to high altitude and is also a feature of tumors.3 It has been demonstrated that physiological shear stress enhances wound closure in cultured human umbilical vein and coronary artery endothelial cells via the action of endothelial cell spreading and migration,4 and cyclic strain stimulates endothelial tube formation, an index of a proangiogenic endothelial phenotype, by a mechanism involving integrins and proteases.5,6 A correlation between blood flow and angiogenesis has also been reported in a variety of animal models7,8 and several in vivo studies support a regulatory role of mechanical stretch in particular, on angiogenesis.9,10 However, details of the process by which hemodynamic force-mediated changes in endothelial cell functions lead to vascular remodeling and angiogenesis remain to be fully elucidated.

The Notch pathway is an evolutionarily conserved intercellular signaling mechanism that is important in vascular development, playing a key role in vascular cell fate decisions.11,12 Notch receptors and ligands are transmembrane proteins that have been identified in mammalian cells. Four Notch receptors (Notch 1 to 4) and 5 ligands (Jagged-1 and -2, δ1, -3, -4) have been identified. Studies using constitutively activated Notch receptors missing their extracellular domains (Notch IC) have shown that Notch signaling determines proliferation, differentiation, and more recently apoptosis in several mammalian cell types.13-15 Notch IC is translocated to the nucleus where it interacts with the CSL family of transcription factors (CBF-1/RBP-Jk, Su (h), and LAG-1) to become a transcriptional activator that can then modulate the expression of Notch target genes that regulate...
cell fate decisions. These include the “Hairy Enhancer of Split” (hes) gene and HES related transcription factors (Hrt’s) that are critically involved in mammalian cell differentiation.14,16

Although Notch receptors are reportedly expressed on vascular endothelial cells17–19 and have been implicated in the process of tumor angiogenesis,20,21 no studies to date have addressed whether the Notch pathway is mechanosensitive in endothelial cells or whether Notch contributes to mediating the hemodynamic force-induced angiogenesis response. Angiopoietin 1 (Ang1), a member of a class of growth factors that activate a receptor tyrosine kinase expressed principally on vascular endothelium i.e., Tie2, is believed to play an important role in the regulation of angiogenesis.22 Here, we report that cyclic strain-stimulated endothelial cell angiogenic activity, as assessed by network formation on matrigel, is mediated in part through a Notch-dependent, Ang1/Tie2 signaling pathway.

Methods

Endothelial Cell Isolation and Culture

Human umbilical vein endothelial cells (HUVECs) were prepared by established methods as previously described.23 HUVECs between passages 2 to 6 were used in all experiments.

Cyclic Strain

For cyclic strain studies, HUVECs were seeded into fibronectin-coated 6-well Bioflex plates (Flexcell International Corp) at a density of ~2×10⁵ cells per well. When cells had reached ~90% confluence, a Flexercell Tension Plus FX-4000T system (Flexcell International Corp) was used to apply a physiological level of cyclic strain to each plate (10% strain, 1 Hz), 0 to 24 hours using the heartbeat simulation as described previously. After strain, HUVECs were harvested for RNA and protein isolation or for use in the network formation/angiogenesis assay.

Network Formation on Matrigel

The formation of a tube-like network by HUVECs on basement membrane matrix Matrigel (BD Biosciences) was analyzed as described previously.25

Notch-Expressing Vectors and Plasmid Preparation

Notch IC expression vector (cytomegalovirus X-Notch 1 IC) was a kind gift of Prof Bettina Kempek, CSF Institute of Clinical Molecular Biology, Neuherberg, Germany. Notch 4 IC expression vector (cytomegalovirus X-Notch 4 IC) was a kind gift from Prof Urban Lendahl, Karolinska Institute, Stockholm, Sweden. Epstein Barr virus-encoded gene product that binds CBF-1 (RPMS-1) was a kind gift from Prof Paul J. Farrell, Ludwig Institute for Cancer Research, Imperial College School of Medicine, London, UK. Plasmids were prepared for transfection according to manufacturer instructions using a Qiagen plasmid Midi Kit (Qiagen) as described previously.26

Endothelial Cell Transfection

Transient transfection of HUVECs was performed using the Gene Pulser Xcell system (Bio-Rad). Cells were transfected with 5 μg of plasmid DNA. Green Fluorescent Protein (GFP) was used as an internal control. For gene silencing studies, 2×10⁵ cells were transfected for 2 μg of siRNA targeting Notch 1, Notch 4, Ang1, Tie1, or Tie2 (Ambion) or a scrambled negative control siRNA (Ambion, cat #:4611) in 75 μL of siRNA electroporation buffer. Transfected cells were allowed to recover for 18 to 24 hours before being exposed to cyclic strain.

Western Blotting

Cell lysates were analyzed for Notch 1 IC, Notch 4 IC, Ang1, Tie1, and Tie2 expression by Western blot. Anti-Notch 1 and Notch 4 antibodies were obtained from Upstate; Anti-Ang1, Tie1, and Tie2 antibodies from Chemicon Int.

Quantitative Real-Time RT-PCR

See supplemental experimental procedures (available online at http://atvb.ahajournals.org).

Statistics

The data shown are the mean±SEM. n=number of individual experiments, with a minimum of 3 independent experiments performed. Statistical significance was estimated using the following analysis: Unpaired Student t test for comparison of 2 groups; Wilcoxon signed rank test for the densitometric data. When >2 groups were present, ANOVA (factorial design) was used (GraphPad Prism). A value of P<0.05 was considered significant.

Results

Cyclic Strain Modulates Notch Signaling in HUVECs

Cyclic strain (10%, 1Hz) caused a temporal regulation of HUVEC Notch 1 and Notch 4 mRNA expression as determined by QRT-PCR with a maximal increase at 4 hours; 21±8 and 23±9 fold increase for Notch 1 and Notch 4, respectively, when compared with unstrained controls (Figure 1a). HUVEC Notch 1 and 4 mRNA levels returned to control levels after 24-hour cyclic strain exposure. Moreover, HUVEC Notch target gene Hrt mRNA levels were temporally increased by cyclic strain with a maximal ~40 fold increase at 4 hours (Figure 1a). Cyclic strain also significantly increased Notch 4 and 1 IC protein expression at 2 and 4 hours, respectively, after strain (Figure 1b). However, by 24 hours, protein levels were significantly decreased.

Cyclic Strain Stimulates HUVEC Network Formation

In response to cyclic strain (10%), network formation by HUVECs on Matrigel was increased in a temporal fashion with significant effects after 4 hours and a maximal effect after 24 hour strain (Figure 2a). The increase in network formation with cyclic strain was concomitant with increased angiopoietin-1 (Ang1), and tyrosine kinase receptor Tie1 and Tie2 mRNA (Figure 2b) and protein (Figure 2c) levels.

Cyclic Strain-Induced Angiogenic Response Is Notch Dependent

Gene silencing of Notch 1 or Notch 4 receptors with specific targeted siRNA duplexes was confirmed at the mRNA and protein level (>70% decrease in Notch 1 and Notch 4 mRNA and protein expression when compared with scrambled control transfected cells, data not shown). Inhibition of Notch 1 and Notch 4 with the respective siRNA attenuated cyclic strain-induced network formation (Figure 3a). Inhibition of endogenous Notch mediated CBF-1/RBP-Jk regulated gene expression using Epstein-Barr virus encoded RPMS-1 also
resulted in a significant decrease in cyclic strain-induced angiogenesis; network length (AU) = 3157 ± 46 versus 1425 ± 20 for control versus RPMS-1 treated under strain conditions (Figure 3b).

Knockdown of Notch 1 or Notch 4 with targeted siRNA inhibited Ang1, Tie1, and Tie2 mRNA expression both under static conditions and after cyclic strain (Figure 4a). Similarly, RPMS-1 inhibited Ang1, Tie1, and Tie2 mRNA under static conditions (Figure 4b). However, RPMS-1 had no effect on strain-induced Ang1, while significantly inhibiting strain-induced Tie1 and Tie2 mRNA levels (Figure 4b).

**Effect of Notch IC Overexpression on Network Formation**

Overexpression of constitutively active Notch 1 and Notch 4 IC in HUVECs was confirmed by Western blot (supplemental Figure 1a, available online at http://atvb.ahajournals.org). In addition, Notch 1 and 4 IC over expression was confirmed by demonstrating a 2- and 2.7-fold increase, respectively, in Notch target gene hrt1 mRNA in these cells compared with mock transfected cells (supplemental Figure 1a). Overexpression of either Notch 1 or 4 IC resulted in a significant increase in network formation by unstrained HUVECs (supplemental Figure 1b). Notch 4 IC, but not Notch 1 IC, overexpression also significantly increased network formation of HUVECs after cyclic strain when compared with mock transfected cells (supplemental Figure 1b). Overexpression of either Notch 1 IC or Notch 4 IC increased Ang1, Tie1, and Tie2 mRNA levels in unstrained HUVECs. Moreover, Notch 4 IC overexpression significantly increased Ang1 and Tie2 mRNA, but not Tie1, in strained HUVECs (supplemental Figure 1c).

**Cyclic Strain-Induced Angiogenic Response Is Ang1/Tie-Dependent**

Gene silencing of Ang1, Tie1, or Tie2 with specific targeted siRNA duplexes was confirmed at the mRNA and protein level (>65% decreases, supplemental Figure IIa). Inhibition of Ang1, Tie1, or Tie2 with the respective siRNA had no effect on network formation by unstrained cells, but significantly inhibited cyclic strain-induced network formation (supplemental Figure IIb).

**Discussion**

Angiogenesis plays a fundamental role in physiology and pathology. It is 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, arthritis, and intraplaque formation.27 Although angiogenesis can be induced by diverse growth factors, increased or decreased blood flow and/or blood pressure is linked with growth of vessels or their regression. Thus, there is intense interest in reaching a better understanding of the relationship between physiological stimuli attributable to blood flow and the clinically important phenomenon of angiogenesis. We report for the first time that cyclic strain stimulates endothelial cells to increase their angiogenic activity by a mechanism mediated by a Notch, Ang1/Tie2 pathway.

We and others have previously shown that Notch receptors and downstream target genes (hes, hrt) are crucial in controlling the modulation of vascular smooth muscle cell (VSMC) growth, migration, and apoptosis in vitro and in vivo.26,28,29 We have also recently reported that cyclic strain modulates Notch receptor signaling in vascular smooth muscle cells.24 However, much less is known about Notch regulation and function in adult endothelial cells. We have previously demonstrated in bovine aortic endothelial cells that the individual hemodynamic forces, shear stress and cyclic strain, are both physiologically relevant stimuli for endothelial cell angiogenic activity.25,5 In our current study, after exposure to physiological levels of cyclic strain, there was a temporal upregulation of Notch 1 and 4 receptor and target gene mRNA and Notch 1 and 4 IC protein in HUVECs. This was concomitant with a time-dependent increase in Ang1, Tie1, and Tie2 expression in these cells and increased angiogenic activity as assessed by network formation on Matrigel.
Moreover, knockdown of Notch 1 or 4 by siRNA, or inhibition of Notch-mediated CBF-1/RBP-Jk regulated gene expression by RPMS-1, which competes at the SKIP/SMART complex of CBF-1, inhibited cyclic strain-induced network formation, whereas overexpression of constitutively active Notch IC enhanced it. Therefore, despite the decrease in Notch IC expression by 24 hours, it is likely that the initial strain-induced increase in Notch signaling is sufficient to cause the sustained increases in the Ang/Tie pathway and the subsequent changes in network formation. Thus, our data demonstrate for the first time that the Notch signaling pathway is mechanoregulated in endothelial cells and that it plays a role in mediating the force-induced angiogenic response of these cells.

The role of Notch signaling in vascular development and angiogenesis has been previously evaluated and various effects reported. Mice deficient for a variety of components of the Notch pathway, including Notch1, Notch1/Notch4, Jagged 1, DLL1, DLL4, Hey1/Hey2, and presenilins resulted in embryonic lethality with vascular remodeling defects. In human, mutations in the Jagged1 and Notch3 genes cause the autosomal dominant disorders Alagille syndrome and CADASIL, respectively, both of which display abnormal vascular phenotypes. The role of Notch signaling in angiogenesis...
has been evaluated by manipulating the expression of different components in endothelial cells. Activation of Notch signaling by ectopic expression of Notch IC or HES1 has been found to result in enhanced network formation of arterial endothelial cells. Notch-induced modulation occurred via an RBP-JK dependent mechanism resulting in the upregulation of several Notch target genes including Hes-1. Moreover, inhibition of RBP-JK dependent Notch signaling in human arterial endothelial cells resulted in attenuation of VEGF-driven network and cord formation in a 3D collagen angiogenesis model. In contrast to this proangiogenic effect of Notch, Leong et al demonstrated that in vitro expression of constitutively activated Notch 4 in human dermal microvascular endothelial cells (HMEC-1) inhibited angiogenesis through β1-integrin–mediated cell matrix interaction and inhibition of endothelial sprouting. Furthermore, although overexpression of a dominant-negative Jagged1 in HUVECs inhibited network formation, addition of an antisense Jagged1 oligonucleotide to bovine microvascular endothelial cells (BMECs) enhanced FGF-dependent invasion and tube formation. These in vitro data suggest that the function of the Notch pathway in endothelial cells is
microvascular ECs indicating that the response we report seems consistent across ECs from different vascular beds and species.

Since their discovery in the 1990s, the angiopoietin family of growth factors has been the focus of growing interest in angiogenesis research. Angiopoietin-1 (Ang1) is a ligand for the Tie2 (tyrosine kinase with immunoglobulin-like loop and EGF homology domains) receptor expressed exclusively on endothelial cells. Besides enhancing endothelial cell migration on fibronectin and collagen in a Tie2-dependent way, Ang1 can also induce endothelial cell adhesion, spreading, focal contact formation, and migration in a Tie2-independent manner through integrins as well as rescue endothelial cells from growth factor deprivation induced apoptosis. Because of the lack of an identified ligand the function of Tie1, which like Tie2 is expressed almost exclusively in vascular ECs, is less clear, although in vivo studies indicate an essential role for Tie1 in vascular development. Indeed, in mice targeted mutations in Tie1, Tie2, or Ang1 all result in embryonic lethality as they fail to form a branched network of small and large vessels. Our data demonstrate that the expression of Ang1, Tie1, and Tie2 is markedly increased in endothelial cells in response to cyclic stretch. Mechanoregulation of Tie1 and Tie2 has been previously reported. Lee et al reported shear stress-induced activation of Tie2, and Chen-Konak et al reported a shear stress–induced downregulation of Tie1.

In both those studies, the maximum response occurred within minutes. Our cyclic strain-induced response was more chronic in nature, with increased expression levels of Ang1 and Tie2 lasting for up to 24 hours, results consistent with those of Chang et al and Zheng et al. Moreover, our data indicate that the cyclic strain-induced increases in Ang1, Tie1, and Tie2 were Notch receptor-dependent as siRNA knockdown of Notch 1 or Notch 4 attenuated the response. To the best of our knowledge, this is the first report of direct regulation of angiopoietins and their Tie receptors by Notch. The cyclic strain-induced increase in Tie1 and Tie2 was dependent on Notch/CBF-1 signaling as evidenced by the marked attenuation by RPMS-1. Although the cyclic strain-induced increase in Ang1 was Notch receptor dependent, it was, however, CBF-1/RBP-Jk independent because RPMS-1 failed to inhibit it, suggesting a mechanistically different regulation by Notch receptors of the ligands and receptors of the Ang/Tie growth factor family after strain. Of interest, evidence for a CBF-1/RBP-Jk-independent Notch signaling pathway also exists. Constitutive overexpression of Notch 1 IC and, to a greater extent, Notch 4 IC, enhanced the strain-induced Ang1 response, whereas only Notch 4 IC over expression increased the strain-induced Tie2 response, and neither affected the strain-induced Tie1 response. Taken together with the siRNA experiments illustrating Notch receptor dependency of the strain-induced Ang1, Tie1, and Tie2 responses, these data suggest that signaling through the full length Notch 1 and

Figure 4. The effect of (a) siRNA-directed knockdown of Notch 1 and Notch 4 and (b) RPMS-1 on cyclic strain-induced Ang1, Tie1, and Tie2 mRNA levels. HUVECs transfected with (a) siRNA targeted to Notch 1 or Notch 4 or with (b) RPMS-1 were exposed to 0% strain (unstrained) or 10% strain (strained) for 24 hours before QRT-PCR analysis of Ang1, Tie1, and Tie2 mRNA levels. Data are the mean±SEM, n=3. *P<0.05 vs unstrained mock transfected, #P<0.05 vs strained mock transfected.
Notch 4 receptor may be of particular importance for Notch regulation of Tie1 whereas Notch IC for both 1 and 4 receptors regulates Tie2 and Ang1 through CBF-1/RBP-Jk–dependent and –independent mechanisms, respectively. Moreover, Notch 4 may be more important than Notch 1 when it comes to Ang1/Tie2 regulation under strain conditions.

Identification of the signaling pathway mediating the effect of cyclic stretch on Notch receptor expression merits further investigation. Of interest, genetic studies in zebrafish and mice have suggested a key role for Notch signaling downstream of VEGF. Given that VEGF regulates Notch,40 and that mechanical stress induces expression of VEGF in several cell types,47,48 the possibility worth examining is that cyclic strain-induced Notch expression may be VEGF-dependent. Nevertheless, our data indicate that cyclic strain stimulates endothelial cell angiogenic activity via a Notch–Ang1/Tie2-dependent pathway. This signaling pathway may thus represent a novel therapeutic target for diseases involving force-induced angiogenesis.

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Disclosures
None.

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Supplementary experimental procedures.

Quantitative real-time RT-PCR (QRTPCR): Total RNA (1-2 µg), isolated from cells using TRIzol™ was reverse-transcribed using iscript™ cDNA Synthesis kit from BIO-RAD (Carlsbad, CA). The gene-specific oligonucleotide sequences were; Notch 1, forward 5' CAGGGTGTGCACCTGAGAT 3', reverse 5' GACAGGCACTCGTTGACATC 3', Notch 4, forward 5’ CTAGGGGCTTTCTCGTCTT 3', reverse 5' CAACTTCTGCCCTTTGCTTC 3', Ang1, forward 5’ GAAGGGAACCGAGCCTATTC 3', reverse 5’ GGGCACATTTGCACATACAG 3’, Tie1, forward 5’ GACTGACCCAGCTTCTGCTC 3’, reverse 5’ CTGCAATCTTGGAGGCTAGG 3’, Tie2, forward 5’ TACACCTGCCTCATGCTCAG 3’, reverse 5’ TTCACAAGCCTTCTCACACG 3’, GAPDH, forward 5’ CGAGATCCCTCCAAAATCAA 3’, reverse 5’ TTCACACCCATGGACGAACAT 3’. For quantitative measurement of mRNA, Real-Time RT-PCR was performed using the Stratagene MX3005 machine and the SYBER green jumpstart PCR kit (Sigma, St. Louis, MO) as described by the manufacturer.

Figure legend for supplemental data.

Figure I: The Effect of Notch 1 and 4 IC over expression on angiogenic activity. (a) Representative Western blots of Notch 1 IC and Notch 4 IC protein (left) and hrt-1 mRNA expression (right) following transfection with constitutively active Notch 1 and Notch 4 IC. Following transfection with or without Notch 1 and 4 IC and exposure to 0% (unstrained) or 10% cyclic strain (strained), (b) angiogenic activity of HUVEC was determined by measuring network formation on Matrigel. Representative images of network formation are shown, together with the cumulative data from three separate experiments. (c) QRTPCR analysis of Ang1, Tie1 and Tie2 mRNA levels following over expression of constitutively active Notch 1
and Notch 4 IC. Data were normalized to GAPDH and the cumulative data represent the mean ± SEM (n=3); *P<0.05 vs unstrained mock transfected, # p<0.05 vs strained mock transfected.

**Figure II:** The effect of siRNA-directed knockdown of Ang1, Tie1 and Tie2 on cyclic strain-induced HUVEC network formation. (a) Representative Western blots for Ang1, Tie1 and Tie2 protein following respective siRNA knockdown. (b) HUVEC transfected with scrambled RNA (control) or with an siRNA targeted to Ang1, Tie1 or Tie2 were exposed to 0% strain (unstrained) or 10% strain (strained) for 24 h before their network formation on Matrigel was determined. Data represent the mean ± SEM (n=3); *P<0.05 vs unstrained mock transfected, # p<0.05 vs strained mock transfected.
Figure I

(a) Mock Transfected | Notch IC Transfected
Notch 1 IC
Notch 4 IC

Fold change in $hrt1$ mRNA Expression
Mock | Notch 1 IC | Notch 4 IC

(b)
Mock | Notch 1 IC | Notch 4 IC
Unstrained
Mock | Notch 1 IC | Notch 4 IC
Strained

Network formation (AU)
Mock | Notch 1 IC | Notch 4 IC
Unstrained
Mock | Notch 1 IC | Notch 4 IC
Strained