Shear Stress Activation of SREBP1 in Endothelial Cells Is Mediated by Integrins

Yi Liu, Benjamin P.-C. Chen, Min Lu, Yi Zhu, Michael B. Stemerman, Shu Chien, John Y.-J. Shyy

Abstract—We investigated the effect of shear stress on the sterol regulatory element–binding protein 1 (SREBP1) in vascular endothelial cells (ECs) and the mechanotransduction mechanism involved. Application of a shear stress (12 dyn/cm²) caused the proteolytic cleavage of SREBP1 and the ensuing translocation of its transcription factor domain into the nucleus. As a result, shear stress increased the mRNAs encoding the low density lipoprotein receptor (LDLR), as well as the binding of 125I-LDL. Using a step flow channel, we showed that SREBP1 activation in ECs under laminar flow is transient, but disturbed flow causes sustained activation. In studying the shear stress–elicited molecular signaling that activates SREBP1, we found that blocking the β₁-integrin with the AIIB2 blocking-type monoclonal antibody inhibited SREBP1 activation induced by shear stress. EC attachment to fibronectin or the activation of β₁-integrin in the suspended ECs by the TS2/16 monoclonal antibody was sufficient for SREBP1 activation. Furthermore, transient transfection assays showed that dominant-negative mutants of focal adhesion kinase and c-Src attenuated the shear stress–increased LDLR promoter activity. These results demonstrate that integrin signaling plays a critical role in the modulation of SREBP in ECs in response to shear stress. (Arterioscler Thromb Vasc Biol. 2002;22:76-81.)

Key Words: shear stress ■ sterol regulatory element–binding protein 1 ■ integrins ■ endothelial cells ■ cholesterol

A key feature of atherosclerosis is lipid accumulation in the artery wall resulting from the transendothelial entry of LDL, followed by LDL oxidation and uptake by monocytes/macrophages. Although every part of the arterial tree is exposed to the same concentration of plasma LDL, atherosclerotic lesions are prevalent in bifurcations and curved regions, which suggests that local hemodynamic forces play a significant role in the focal nature of the lesions. This thesis is supported by studies demonstrating that LDL infused into experimental animals is preferentially located in the areas with disturbed blood flow, such as branch points. The increased LDL uptake in the lesion-prone areas can be due to an enhanced permeability through endothelial junctions and/or an LDL receptor (LDLR)–mediated endocytosis. Using an in vitro flow system with cultured vascular endothelial cells (ECs), Sprague et al demonstrated that laminar shear stress increased the binding, internalization, and degradation of LDL in ECs, and these changes were mediated through upregulated LDLR. To date, the molecular mechanism by which shear stress modulates the expression of LDLR has not been elucidated.

Sterol regulatory element–binding proteins (SREBPs) are key transcription factors that modulate the multiple genes involved in the biosynthesis of cholesterol and fatty acids and receptor-mediated LDL uptake. There are 3 members in the SREBP family: SREBP1a and SREBP1c are encoded by the same gene, whereas SREBP2 is from a separate gene (see Brown and Goldstein for a review). When cells are depleted of sterols, the SREBP cleavage-activating protein (SCAP) escorts SREBPs from the endoplasmic reticulum to the Golgi, where the SREBPs are subjected to a 2-step proteolytic cleavage by S1P and S2P. As a result, the N-terminal transcription factor domain of SREBPs translocates into the nucleus and acts on the sterol regulatory element (SRE), which activates target genes such as LDLR and 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) synthase involved in cholesterol metabolism, as well as fatty acid synthase and acetyl CoA carboxylase involved in lipogenic pathways.

Several laboratories, including ours, have demonstrated that shear stress can regulate gene expression, thus modulating endothelial biology (see Davies and Chien for reviews). Kinases in both the cytoplasm (eg, mitogen-activated protein kinases) and focal adhesions (eg, focal adhesion kinase [FAK] and c-Src) have been found to modulate the shear stress–responsive genes. It appears that integrins, by functioning as shear stress sensors, can regulate these kinases. The extracellular domain of integrins binds to various extracellular matrix (ECM) proteins such as fibronectin, whereas the cytoplasmic domain interacts with kinases in the focal adhesions and with cytoskeletal proteins (eg, actin filaments). The unique structural features of integrins enable

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them to function not only as adhesion receptors and signaling regulators of cytoskeletal organization but also as mechano-sensors to transduce the signals elicited by shear stress.\textsuperscript{15}

The aim of the current study was to explore the molecular mechanism underlying the shear stress–modulated SREBP in ECs. We found that shear stress activates SREBP1, that disturbed flow can prolong SREBP1 activation, and that shear stress regulation of SREBP1 is mediated by integrins and the associated kinases in the focal adhesion.

Methods

Cell Culture

Bovine aortic endothelial cells (BAECs) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 2 mmol/L L-glutamine, and 1 mmol/L each of penicillin-streptomycin and sodium pyruvate. Cell cultures were maintained in a humidified 95% air–5% CO\textsubscript{2} incubator at 37°C.

Shear Stress Experiments

A parallel-plate flow channel was used to impose laminar shear stress on cultured ECs, as previously described.\textsuperscript{16} DMEM supplemented with 20% FBS was used to perfuse the ECs. A laminar shear stress of 12 dyn/cm\textsuperscript{2} was generated by the flow resulting from a hydrostatic pressure difference between a high and a low reservoir. The flow system was kept at 37°C and maintained in a humidified air with 5% CO\textsubscript{2}.

The step flow channel was constructed according to previous reports.\textsuperscript{17,18} Disturbed flow was created by a step expansion of the flow-reattachment area of the step flow channel, the shear stress was zero but the spatial gradient was high, whereas at the downstream laminar flow area the shear stress was high with no spatial gradient.

Immunoblotting Analysis

BAECs were lysed by a standard lysis buffer, the cell lysates were subjected to 10% SDS–polyacrylamide gel electrophoresis (PAGE), and the proteins in the gel were transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk and then incubated with 2%BSA anti-FAK mAb (Santa Cruz Biotechnology), followed by immunoblotting with use of a goat anti-mouse horseradish peroxidase conjugate (Santa Cruz Biotechnology). To detect tyrosine phosphorylation of FAK, it was first immunoprecipitated from cell lysates by the Lowry procedure (Bio-Rad).

Immunostaining Analysis

BAECs were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS, and blocked with 1% bovine serum albumin in PBS. The fixed cells were incubated in PBS containing a polyclonal anti-SREBP1 (Santa Cruz Biotechnology) at a concentration of 1 μg/mL and then incubated with a fluorescein isothiocyanate–conjugated anti-rabbit IgG (Santa Cruz Biotechnology). The stained specimens were photographed under a Nikon TE 300 inverted microscope with fluorescein isothiocyanate excited at 488 nm and detected within a band between 506 and 538 nm.

Treatment With mAbs

Ten micrometers of TS2/16 mAb purified from hybridoma supernatants (American Tissue Culture Collection) or mouse IgG (Chemicon) was mixed with 40 μL latex beads (Sigma) in 0.5 mL DMEM containing 20% FBS at 37°C for 3 hours. The beads coated with TS2/16 or mouse IgG were then added to 2×10\textsuperscript{6} BAECs suspended in 0.5 mL DMEM containing 20% FBS. After incubation on a rotary shaker, cells were lysed for immunoblotting analysis of SREBP1 cleavage and FAK tyrosine phosphorylation. In β\textsubscript{3}-integrin–blocking experiments, BAECs seeded on slides coated with fibronectin were incubated with DMEM containing 20% FBS, 10 μg AIB2 mAb (Developmental Studies Hybridoma Bank, University of Iowa), or mouse IgG at 37°C for 3 hours before shear stress experiments.

Transient Transfection and Luciferase Activity Assay

Plasmid pLDLR-Luc is a luciferase reporter driven by the LDLR promoter,\textsuperscript{21} and plasmid pSV–β-galactosidase (β-gal) contains a β-gal gene driven by the simian virus 40 promoter. pLDLR-Luc (3.5 μg per slide) and pSV–β-gal (0.5 μg per slide) together with dominant-negative mutants of FAK, c-Src, SCAP, or pcDNA3 (0.5 μg per slide) were transiently transfected into BAECs cultured on glass slides by use of the Lipofectamine method (Gibco BRL). Twenty-four hours after transfection, the BAEC monolayers were subjected to shear stress or kept under static conditions. The cells were lysed in a buffer containing 0.1 mol/L potassium phosphate, pH 7.9, 0.5% Triton X-100, and 1 mmol/L diithiothreitol to release the Luc and β-gal for their activity assays. The numerical readings from the Luc assay were normalized to those of the β-gal assay.

Results

Shear Stress Activation of SREBP1 in BAECs

We investigated whether shear stress modulates SREBP1 in ECs. Monolayers of BAECs were either kept as static controls or subjected to laminar flow at a shear stress of 12 dyn/cm\textsuperscript{2} with the use of fresh DMEM containing 20% FBS as the perfusing medium. SREBP1 in cell lysates prepared from static or sheared cells was analyzed by immunoblotting. As shown in Figure 1A, shear stress induced the cleavage of SREBP1, as previously described.\textsuperscript{20} In brief, cells were chilled and then incubated with DMEM containing 2 μg/mL \textsuperscript{125}I-LDL in the presence or absence of a 50- or a 100-times molar excess of unlabeled LDL at 4°C for 1 hour. The cells were then washed with ice-cold phosphate-buffered saline (PBS). After dissolution in 0.1N NaOH, an aliquot of cell suspension was used to determine the amount of radiolabeled \textsuperscript{125}I-LDL, and another aliquot was used to determine the amount of cellular proteins by the Lowry procedure (Bio-Rad).

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increased to levels comparable to those in their respective static controls. As a control, BAECs were incubated with DMEM containing 20% lipoprotein-deficient serum. The activation of SREBP1 was sustained in BAECs cultured under lipoprotein-deficient serum for at least 24 hours (Figure 1B). Furthermore, shearing media containing lipoprotein-deficient serum supplemented with 25-hydroxycholesterol also caused the transient activation of SREBP1 (Figure 1C), which indicates that shear stress activation of SREBP1 is independent of the level of extracellular sterols.

Shear Stress Increases LDLR mRNA and LDL Binding

To investigate whether shear stress activation of SREBP1 would result in an SRE-mediated transcriptional activation, we investigated whether shear stress regulates the expression of the transcripts encoding LDLR, an SREBP target gene. RT-PCR analysis of RNA samples collected from static and sheared BAECs showed that the application of shear stress induced a transient upregulation of LDLR mRNA in BAECs (Figure 2A). In contrast, shear stress had little effect on inducing HPRT, a housekeeping gene.

To correlate the temporal changes in LDLR mRNA with LDL metabolism, we investigated whether shear stress regulates a transient increase in LDL binding to ECs. Monolayers of BAECs were either kept as static controls or subjected to a laminar shear stress for various lengths of time, followed by 125I-LDL binding assays. 125I-LDL binding to BAECs that had been sheared for 4 hours was 1.4 ± 0.1 times more than that in static cells (Figure 2B). The binding increased to 1.7 ± 0.3 times after shearing for 8 hours but returned to a level similar to that in static controls after shearing for 48 hours. In static experiments in which BAECs were exposed to DMEM containing 20% lipoprotein-deficient serum, the binding of 125I-LDL remaining was determined. The relative 125I-LDL binding is defined as the amount of 125I-LDL bound to the sheared or lipoprotein-deficient serum–treated cells compared with that of static controls. Bars represent mean ± SD from 4 independent experiments. Asterisks indicate significance difference (P < 0.05) between sheared and static controls.
dyn/cm² could result from a sharp, temporal gradient of shear stress. After the ECs adapted to the applied laminar flow, SREBP1 became quiescent. To test whether the constant presence of a shear stress gradient due to disturbed flow can cause sustained SREBP1 activation, ECs were subjected to different flow patterns in a step flow channel. We compared the subcellular localization of SREBP1 in cells under laminar versus disturbed flows by use of immunostaining. As shown in Figure 3, SREBP1 translocated into nuclei in cells under both laminar and disturbed flows for 1 hour, indicating SREBP1 activation. The percentage of cells showing SREBP1 activation under laminar flow (52.6±9.4%) was comparable to that under disturbed flow (47.5±5.6%). Continuing to shear the cells with laminar flow at 2 and 9 hours led to a progressive decrease in SREBP1 activation. Prolonged shearing under laminar flow for 12 hours led to elongated BAECs, and only 14.3±5.3% of the cells revealed SREBP1 staining in the nuclei. However, the percentages of SREBP-activated cells under disturbed flow for 2, 9, and 12 hours were similar to that for 1 hour.

### Integrons Regulate SREBP1 Activation

Integrons containing the β₁-subunit (eg, α₁β₁), by interacting with cognate ECM proteins (eg, fibronectin), can function as mechanosensors. If integrins mediate shear stress activation of SREBP1, then blocking the integrins that interact with ECM proteins should inhibit such activation. To test this hypothesis, BAECs were treated with AIIB2 anti-β₁ mAb, which inhibits cell attachment to fibronectin, laminin, and collagen. In parallel experiments, cells were treated with mouse IgG as isotype controls. As shown in Figure 4, shear stress caused SREBP1 cleavage in control cells treated with mouse IgG, but this activation was attenuated when the cells were incubated with AIIB2 mAb.

Attaching suspended cells to various ECM proteins has been widely used as an experimental approach to study integrin-mediated signal transduction. As an alternative approach, we investigated whether attaching BAECs to fibronectin would lead to SREBP1 activation. As shown in Figure 5A, attaching cells to fibronectin caused a transient increase in the level of the 68-kDa mature form of SREBP1. The amount of cleaved SREBP1 peaked at 1 hour and returned to basal level at 8 hours. This time course of cleaved SREBP1 was similar to that induced by shear stress (Figure 1). In contrast, SREBP1 levels changed little in cells attached to poly-L-lysine for up to 2 hours.

Another way to activate integrins is to conjugate cells in suspension to integrin-activating mAbs that "lock" integrins in their active conformation. Thus, we examined whether attaching BAECs to fibronectin would lead to SREBP1 activation. As shown in Figure 5B, such treatment caused the cleavage of SREBP1 in BAECs, which indicates that integrin activation is sufficient for SREBP activation. As a positive control, β₁-activation by TS2/16 mAb also induced the tyrosine phosphorylation of FAK in ECs. The results in Figures 4 and 5 indicate that...
The major findings in the present study are as follows: (1) shear stress activates SREBP1 in ECs in the presence of sterols; (2) laminar flow activates SREBP1 in a transient manner, whereas disturbed flow causes sustained SREBP activation; and (3) shear stress activation of SREBP1 is mediated by integrins. The activation of SREBP1 by shear stress would result in the transcriptional activation of genes regulated by the SRE, such as the LDLR gene (Figure 2). Regulation of SRE-mediated genes by shear stress also includes the HMG-CoA synthase and fatty acid synthase genes (data not shown). SREBP1a and SREBP2 appear to be more able to activate genes involved in cholesterol metabolism; however, SREBP1c preferentially regulates genes engaged in fatty acid synthesis. Antibodies used in the current study could not distinguish SREBP1a from SREBP1c. Because shear stress can regulate gene products involved in cholesterol (e.g., LDLR) or lipogenic (e.g., fatty acid synthase) pathways, it is likely that both SREBP1a and SREBP1c, together with SREBP2, are all activated by shear stress.

The proteolytic cleavage of SREBPs is mainly regulated by the cellular level of sterols, and sterol sensitivity is controlled by SCAP. In sterol-depleted cells, SCAP interacts with the regulatory domain (C-terminal domain) of SREBPs to form a complex for escorting SREBPs from the endoplasmic reticulum to the Golgi. The regulation of SRE-mediated genes by SREBP in response to shear stress is supported by the inhibition of shear stress–induced LDLR promoter activity by the dominant-negative mutant of SCAP (Figure 6C). Most in vitro experiments involving SREBPs were performed in fibroblasts, Chinese hamster ovary cells, and hepatocytes. To date, there has been no documented report on SREBP regulation in ECs, even though the dysfunction of cholesterol metabolism and the ensuing lipid deposition in vascular cells have important pathophysiological significance. It is unlikely that the shear stress activation of SREBP1 in our study was due to the depletion of extracellular sterols, because BAECs were perfused by fresh DMEM containing 20% FBS, a condition with an ample level of sterols. This notion is

**FAK and c-Src Regulate SREBP1 in Response to Shear Stress**

Tyrosine kinases in the focal adhesions associate with integrin-elicited signal transduction and are involved in the mechanotransduction pathways. Using dominant-negative mutants of FAK and c-Src, we investigated further whether these kinases also regulate the shear stress induction of LDLR. Compared with static controls, BAECs transfected with pcDNA3 showed 3.8 ± 0.3 times greater induction of Luc activity under shear stress (Figure 6). Cotransfection of either FAK-Y397F or Src-K297N, the dominant-negative mutants of FAK and c-Src, respectively, drastically reduced the Luc induction by shear stress to 1.7 ± 0.4 and 1.4 ± 0.1 times greater, respectively. These results suggest that FAK and c-Src are involved in the shear stress induction of SREBP.

To correlate SREBP activation with LDLR gene expression in response to shear stress, we investigated whether the shear stress–induced LDLR promoter activity would also be blocked by SCAP-C, which encodes a truncated form (C-terminus) of SCAP and has been shown to block SREBP translocation from the endoplasmic reticulum to the Golgi and hence, impair SREBP activation. Cotransfection of SCAP-C with LDLR-Luc reduced the shear stress induction to 1.2 ± 0.2 times. This result indicates that shear stress–induced LDLR gene expression is mediated through SREBP.

**Discussion**

The results are mean ± SD from 3 independent experiments.
supported by the finding that shear stress activates SREBP1, even in the presence of 25-hydroxycholesterol, an agent that is commonly used to suppress SREBP activation.

The attachment of BAECs to fibronectin had the same effect as did shear stress in activating SREBP1 (Figure 5A). Fibronectin is an ECM ligand for many β1-containing integrins, such as αvβ3, αvβ5, and αvβ6. This result suggests that integrin activation plays a critical role in SREBP activation after cell attachment, as does shear stress application. The β-cytoplasmic tails of integrins are important for adhesion, spreading, and migration of cells on the ECM.27 Our data suggest that β1-integrin is important in SREBP activation, because mAb activation of β1 caused SREBP1 cleavage, whereas the blocking-type anti-β1 attenuated the shear stress activation of SREBP1. β1 Occupancy by the RGD peptide ligand has also been shown to be important for mechanotransduction (eg, protein kinase A signaling) in ECs in response to magnetic twisting stress.28 Shear stress may act on the β1-integrin/ECM to cause SREBP1 activation.

In our experiments, BAECs were subjected to laminar flow with Reynolds’ numbers in the order of 103.17 The transient activation of SREBP1 by such a laminar shear stress can be viewed as a response to the step change of shear stress from 0 to 12 dyn/cm², which represents a sharp, temporal gradient of shear stress.29 With continuous exposure to a constant level of laminar shear stress at 12 dyn/cm², ECs can adapt to the constant flow that no longer has a temporal or spatial gradient of shear stress. The potential roles of high- versus low-shear stress gradients in vascular biology are flow patterns in the lesion-prone areas that are disturbed by high spatial and temporal shear stress gradients and flow patterns in lesion-resistant areas being steady laminar flows with low-shear stress gradients.30,31 Thus, our study supports the hypothesis that the focal distribution of lipids in the arterial tree is due in part to local flow patterns.

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