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Shear Stress–Dependent Regulation of the Human β-Tubulin Folding Cofactor D Gene

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Abstract—The flowing blood generates shear stress at the endothelial cell surface. The endothelial cells modify their phenotype by alterations in gene expression in response to different levels of fluid shear stress. To identify genes involved in this process, human umbilical vein endothelial cells were exposed to laminar shear stress (venous or arterial levels) in a cone-and-plate apparatus for 24 hours. Using the method of RNA arbitrarily primed polymerase chain reaction, we cloned a polymerase chain reaction fragment representing an mRNA species downregulated by arterial compared with venous shear stress (shear stress downregulated gene-1, SSD-1). According to Northern blot analysis, corresponding SSD-1 cDNA clones revealed a similar, time-dependent downregulation after 24 hours of arterial shear stress compared with venous shear stress or static controls. Three SSD-1 mRNA species of 2.8, 4.1, and 4.6 kb were expressed in a tissue-specific manner. The encoded amino acid sequence of the human endothelial SSD-1 isoform (4.1-kb mRNA species) revealed 80.4% identity and 90.9% homology to the bovine β-tubulin folding cofactor D (tfcD) gene. Downregulation of tfcD mRNA expression by shear stress was defined at the level of transcription by nuclear run-on assays. The tfcD protein was downregulated by arterial shear stress. The shear stress–dependent downregulation of tfcD mRNA and protein was attenuated by the NO synthase inhibitor *N*ω-nitro-L-arginine methyl ester. Furthermore, the NO donor DETA-NO downregulated tfcD mRNA. Because tfcD was shown to be a microtubule-destabilizing protein, our data suggest a shear stress–dependent regulation of the microtubular dynamics in human endothelial cells. **(*Circ Res*. 2000;87:1188-1194.)**

Key Words: endothelial cells ■ shear stress ■ RNA arbitrarily primed polymerase chain reaction ■ β-tubulin folding cofactor D ■ nitric oxide

The flowing blood generates shear stress at the endothelial cell surface. Physiological shear stress is not only involved in the regulation of vascular tone but is also considered a protective mechanism against the localization of arteriosclerotic plaques.[1,2]

The mean physiological shear stress acting on endothelial cells is higher in arterial vessels (≈15 dyne/cm^2) compared with venous vessels (≈1 dyne/cm^2). This difference in shear stress affects the shape and differentiation of endothelial cells in arteries and veins. Endothelial cells align their shape and reorganize their cytoskeleton in response to the direction and degree of shear stress.[3,4] Long-term application of arterial levels of laminar shear stress results in alignment of actin stress fibers in the direction of flow.[5] However, the molecular mechanism underlying this cytoskeletal reorganization is not well understood. Furthermore, the regulation of gene expression by arterial levels of shear stress could be mediated by a mechanism involving vasoactive substances released from endothelial cells in a flow-dependent manner. High shear stress stimulates flow-dependent dilation of large vessels[6,7] by release of endothelium-derived NO in animal studies and induces endothelial cell NO synthase expression.[8–10]

In view of these differences, we compared the gene expression of human endothelial cells exposed to high arterial or low venous levels of shear stress by RNA arbitrarily primed (RAP) polymerase chain reaction (PCR). One cDNA species showing very strong regulation by high shear stress was cloned and studied in more detail. We also tested the hypothesis that shear stress–dependent regulation of this gene may be mediated by NO.

Materials and Methods

Cell Culture and Application of Shear Stress

All cell culture reagents and chemicals were purchased from Sigma Chemical Co if not indicated otherwise. Human umbilical vein endothelial cells (HUVECs) were isolated as described previously.[11,12] HUVECs were subjected to laminar shear stress in a cone-and-plate viscometer[13] with minor modifications as described.[14] Laminar shear stress of 1 dyne/cm^2 (0.1 N/m^2 [venous or low shear stress]) or 15 or 30 dyne/cm^2 (1.5 or 3 N/m^2, respectively; arterial or high shear stress) was applied in a humidified environment with 5%

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CO_2 at 37°C. Cell culture medium containing dextran did not affect mRNA expression in this study (Figure 1 online; available in the online data supplement at http://www.circresaha.org). Shear stress did not increase medium temperature or lactate dehydrogenase release (Figures 2 and 3 online).

RNA Isolation and RAP-PCR

Total RNA from endothelial cells was isolated by guanidinium thiocyanate/cesium chloride centrifugation.[15] The mRNA was then obtained using an mRNA purification kit (Pharmacia).

RAP-PCR was used to identify differentially expressed transcripts (RAP-PCR kit, Stratagene).

Northern Blot Analysis

Northern blot analysis was done as previously described.[16] The human multiple tissue Northern blot was purchased from Clontech. The expression of control gene GAPDH was not affected by laminar shear stress (Figure 4 online).

cDNA Cloning and DNA Sequence Analysis

Selected RAP-PCR fragments were cloned with the pCR-Script Amp SK(+) cloning kit (Stratagene). Single clones showing an identical shear stress–dependent regulation by Northern blot analysis were used to screen 1×10^6 plaque-forming units of an oligo(dT) and randomly primed human heart–Lambda ZAP II–cDNA library (Stratagene).

The cDNA sequence was determined by cycle sequencing on an automated ABI PRISM 373A DNA sequencer (ABI/Perkin Elmer). The DNA and deduced protein sequence was analyzed by database searches of GenBank.[17]

Nuclear Run-On Assays

Nuclear run-on assays were performed as previously described.[18]

Inhibitor and NO Donor Studies

HUVECs were cultured under static conditions or exposed to laminar shear stress for 24 hours with or without the NO synthase inhibitor *N*ω-nitro-L-arginine methyl ester (L-NAME, 1 or 400 μmol/L).

In further studies, static cultures of HUVECs were incubated with the NO donor DETA-NO (0.1 to 2 mmol/L) for 24 hours and analyzed by Northern blotting.

Production of Antibodies Directed Against Shear Stress Downregulated Gene-1 (SSD-1) Peptide

One strongly immunogenic peptide of SSD-1 protein was selected using Gene Runner software (Hastings Software, Inc). A 14-mer immunogenic peptide (VKKEIKNSKDIQKL, SSD-1 residues 1075 to 1088) was synthesized and conjugated with an additional N-terminal cysteine residue to KLH and used for immunization of rabbits (Eurogentec, Berlin, Germany). Specificity of preimmune and SSD-1 antiserum was tested in slot blot and Western blot analysis.

Protein Isolation and Western Blot Analysis

Protein isolation and Western blot analysis using SSD-1 antiserum was performed as described.[16]

Statistics

Data are given as mean±SEM (n≥3 in all cases). Statistical analysis was performed with ANOVA procedure followed by the Bonferroni *t* test (multiple comparison) or the Student *t* test (SigmaStat software, Jandel Corp). Differences were taken as statistically significant at $P<0.05$.

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

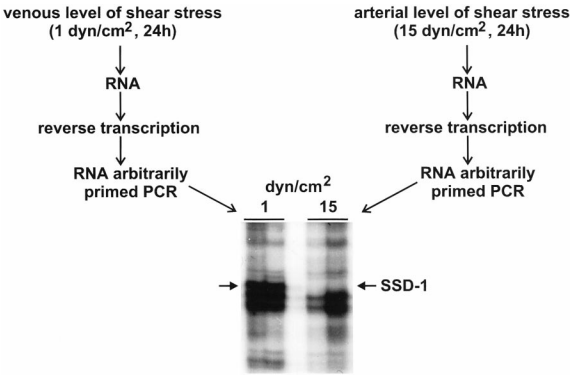

Figure 1. Identification of SSD-1 by RAP-PCR. HUVECs were subjected to laminar shear stress of 1 dyne/cm^2 (mean shear stress in venous vessels) or 15 dyne/cm^2 (mean arterial shear stress) for 24 hours in a cone-and-plate apparatus. Poly(A)$^+$ RNA was isolated from 2 independent experiments for each level of shear stress and analyzed by RAP-PCR. One RAP-PCR fragment downregulated by laminar arterial shear stress, compared with venous shear stress, is indicated by an arrow. The corresponding cDNA was termed SSD-1.

Results

Molecular Cloning of SSD-1, a Gene Downregulated by Arterial but Not Venous Shear Stress

After exposure of HUVECs to low venous levels of shear stress (1 dyne/cm^2) or high arterial levels of shear stress (15 dyne/cm^2) for 24 hours, 30 cDNA fragments differentially expressed at these 2 levels of shear stress were isolated by the method of RAP-PCR. Using this unbiased approach, we selected one mRNA species showing the greatest downregulation by long-term (24 hours) shear stress of 15 dyne/cm^2 (Figure 1). We termed this mRNA species "shear stress downregulated gene-1" (SSD-1). SSD-1 RAP-PCR fragments were cloned into the pCR-Script Amp SK(+) plasmid.

Single SSD-1 clones were tested by Northern blot analysis of HUVECs exposed to shear stress of 1 or 15 dyne/cm^2 (24 hours). SSD-1 clones showing a similar downregulation by shear stress of 15 dyne/cm^2 were used to isolate 3 independent, overlapping cDNA clones from a human heart–Lambda ZAP II–cDNA library. A SSD-1 fragment present in all 3 cDNA clones was hybridized with RNA of HUVECs exposed to shear stress of 15 dyne/cm^2 for varying periods of time (Figure 2). This SSD-1 fragment detects an RNA transcript of 4.1 kb in HUVECs. The endothelial SSD-1 transcript was downregulated by shear stress of 15 dyne/cm^2 for 24 hours by 70% as compared with the static control. In contrast, long-term application of low venous shear stress (1 dyne/cm^2, 24 hours) had no effect on SSD-1 mRNA expression. Independently, in separate experiments, we isolated fragments with sequence identity to SSD-1 showing a similar downregulation by long-term arterial shear stress compared with venous shear stress or static control (not shown).

SSD-1 Encodes Human β-Tubulin Folding Cofactor D (tfcD)

The DNA sequence of 3 SSD-1 cDNA clones suggested that these clones originated from the same gene because of large

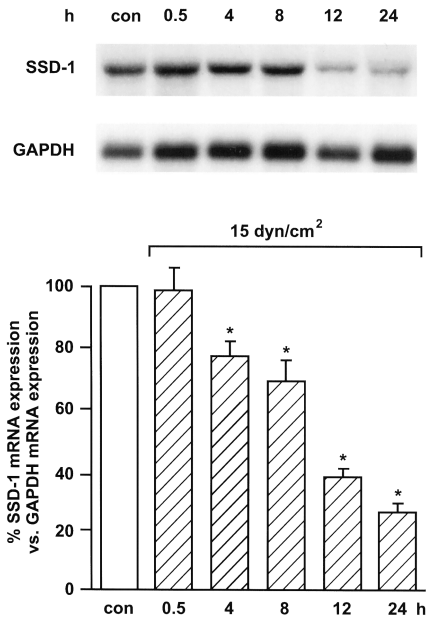

Figure 2. Time course of SSD-1 mRNA expression in response to arterial laminar shear stress. HUVECs were exposed to laminar shear stress of 15 dyne/cm^2 (mean shear stress in arterial vessels) in a cone-and-plate apparatus for the times indicated (con indicates control without shear stress). Total RNA was harvested, and 10-μg samples were subjected to electrophoresis and Northern blotting and hybridized to cDNA probes for SSD-1 and GAPDH. Results shown are representative of 4 independent experiments. In the corresponding bar graph, SSD-1 mRNA expression is normalized vs GAPDH expression and indicated as percentage of expression under static culture conditions (static condition, white bar; shear stress, dashed bars). *$P<0.05$ vs control.

overlapping sequences. The sequence of the SSD-1 cDNA fragments revealed 4049 bp with a 3744-bp open reading frame encoding a protein of 1248 amino acids (Figure 3). The SSD-1 amino acid sequence revealed 2 putative cell adhesion sequences (RGD sites) and 9 potential *N*-myristoylation sites. We found in the SSD-1 protein putative phosphorylation sites for cAMP- and cGMP-dependent protein kinase (1 site), tyrosine kinase (1 site), protein kinase C (8 sites), and casein kinase II (22 sites).

The SSD-1 amino acid sequence revealed 80.4% identity and 90.9% homology to the recently cloned bovine tfcD.[19] Alignment of the amino acid sequences of human SSD-1 encoding tfcD with bovine tfcD is available in the online data supplement at http://www.circresaha.org (Figure 5 online).

Tissue-Specific Expression of Human tfcD Gene

The human tfcD was hybridized to RNA from different human tissues (Figure 4). The tfcD probe detected mRNA species of 4.6, 4.1, and 2.8 kb. The 4.1-kb transcript, found in endothelial cells, was the most abundant tfcD mRNA in all of the human tissues we tested (heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas). In addition, 2 tfcD mRNA species of 4.6 and 2.8 kb, expressed at lower levels, were detected in human heart, brain (4.6 kb only), placenta, lung (2.8 kb only), skeletal muscle (2.8 kb only), and pancreas, suggesting the presence of tissue-specific tfcD isoforms.

Transcriptional Regulation of tfcD mRNA by Shear Stress

Downregulation of tfcD mRNA by shear stress could be due to downregulation at the transcriptional level or to decreased mRNA stability. Therefore, we performed nuclear run-on assays with nuclei isolated from HUVECs with or without application of arterial levels of laminar shear stress. Using a new reverse transcriptase (RT)–PCR–based run-on assay, downregulation of tfcD mRNA expression (to 11±7% of the level in static controls; n=3, $P<0.001$) by arterial levels of shear stress was found at the level of transcription (Figure 5). As a control, endothelial NO synthase (eNOS), a gene well known to be upregulated by laminar shear stress, was induced at the transcriptional level in the same nuclei (to 273±61% of the level in static controls; n=3, $P<0.05$), whereas mRNA expression of the housekeeping gene GAPDH was not affected by shear stress. RT-PCR fragments amplified with this procedure reflect de novo mRNA synthesis in isolated nuclei during the incubation period because RT-PCR fragments were usually not detectable or were less abundant (maximum 20% of control) in control nuclei lysed immediately after isolation.

Downregulation of tfcD by Arterial Levels of Shear Stress Is Mediated by a NO-Dependent Pathway

Because arterial shear stress induces endothelial NO synthesis, the effect of eNOS inhibition (L-NAME, 400 μmol/L) on shear stress–dependent downregulation of tfcD mRNA was tested (Figure 6). The inhibitor had no significant effect on basal tfcD mRNA expression but prevented the downregulation of tfcD mRNA by arterial shear stress.

A similar downregulation of tfcD protein was observed after exposure to different levels of arterial shear stress (15 dyne/cm^2, 47.6±6.7%; 30 dyne/cm^2, 40.6±5.1%; n≥5 each, $P<0.05$ versus static control in each) (Figure 7), whereas application of long-term venous shear stress (1 dyne/cm^2, 24 hours) did not affect tfcD protein expression. The downregulation of tfcD protein expression by arterial levels of shear stress was attenuated by eNOS inhibition with L-NAME (400 μmol/L) (Figure 7).

To get direct evidence for NO-dependent regulation of tfcD expression, HUVECs were incubated under static conditions with different concentrations of the NO donor DETA-NO for 24 hours (Figure 8). At DETA-NO concentrations higher than 1 mmol/L, tfcD mRNA was downregulated by 30% as compared with control.

Discussion

Laminar shear stress induces a variety of molecular changes in endothelial cells.[2,4] Cellular changes in response to shear stress include alignment of cells in the direction of flow, reorganization of the cell surface, downregulation of fibronectin expression, and increased mechanical stiffness.[4]

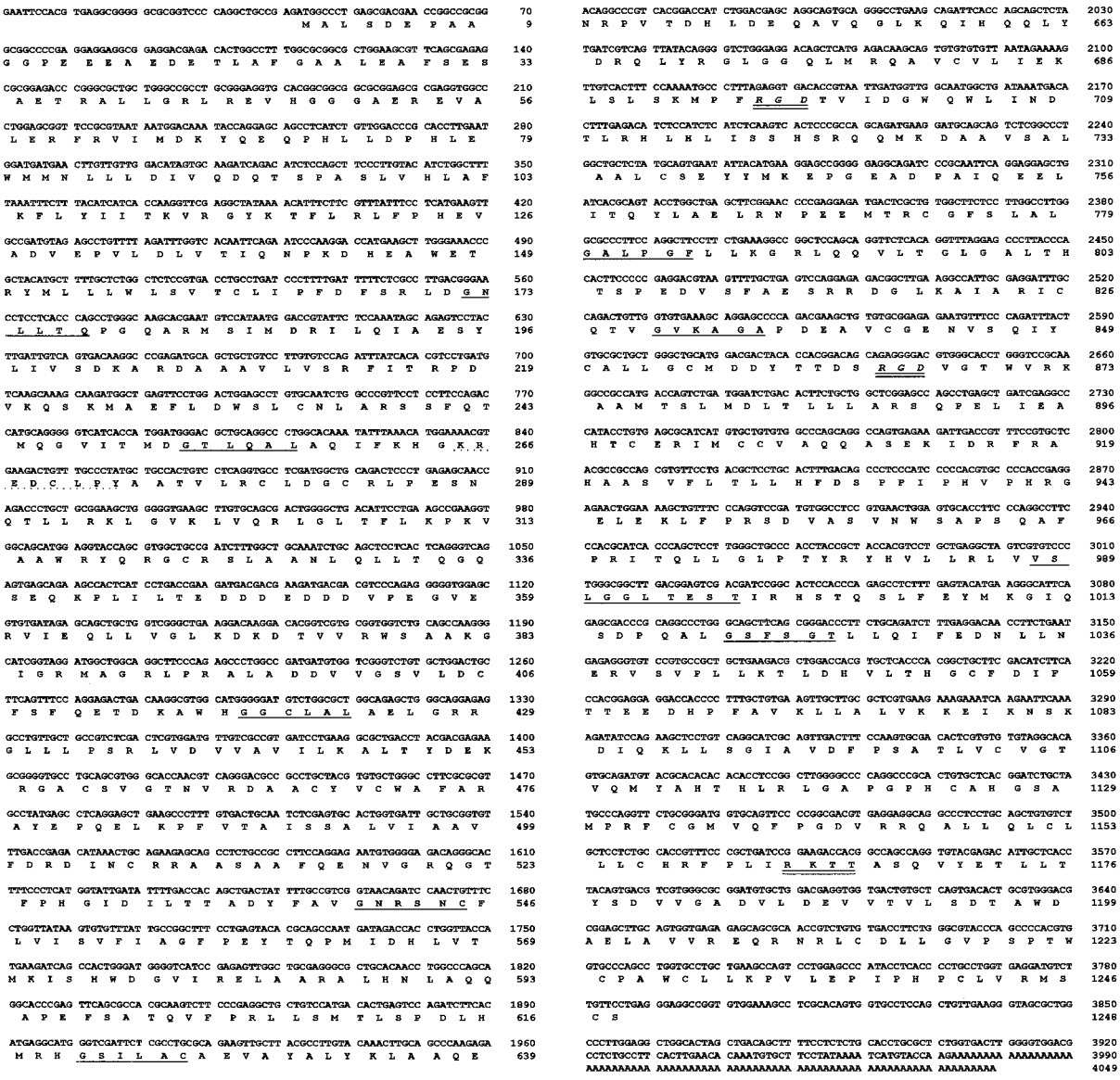

Figure 3. Nucleotide and deduced amino acid sequence of SSD-1 identified as tfcD. The putative amino acid sequence of SSD-1/tfcD protein is shown in one-letter code below the nucleotide sequence. Numbers on the right indicate positions of nucleotides and amino acids. The position of putative cell adhesion sequences (RGD sites), *N*-myristoylation sites, and phosphorylation sites for cAMP- and cGMP-dependent protein kinase and tyrosine kinase are indicated. The sequence reported in this paper has been deposited in the EMBL/GenBank database (accession No. AJ006417).

Our interest was to identify changes in endothelial gene expression by long-term exposure to venous or arterial levels of laminar shear stress. We initially identified 30 cDNA fragments differentially expressed after application of venous or arterial shear stress in endothelial cells. However, for an overall estimation of the number of genes differentially expressed by long-term venous or arterial levels of laminar shear stress, initially identified fragments have to be confirmed by isolation of individual cDNA clones and alternative techniques (eg, subtractive hybridization, cDNA expression array). We focused in this study on one gene with apparently the most prominent downregulation by arterial shear stress. This gene was identified as the human tfcD gene.

The recently cloned bovine tfcD is involved in the generation of exchange-competent β-tubulin.[19] The folding of newly synthesized β-tubulin into its native conformation is the first posttranslational step in the pathway leading to the tubulin heterodimer (consisting of one α- and one β-tubulin polypeptide). The correct folding of α- and β-tubulin requires the assistance of a cytosolic chaperonin and several additional tubulin folding cofactors (A through E).[20,21] This tubulin-specific chaperone/tfc supercomplex is considered as a dimer-making machine.[20] Because tubulin generates the microtubules as essential elements of the cytoskeleton, this process might affect the reorganization of the cytoskeleton in response to arterial laminar shear stress. Apart from folding β-tubulin, tfcD can capture β-tubulin in tfcD/β-tubulin complexes by disrupting native αβ-tubulin heterodimers.[22] Recently, tfcD was shown to modulate microtubule dynamics by sequestering β-tubulin from GTP-bound αβ-tubulin het-

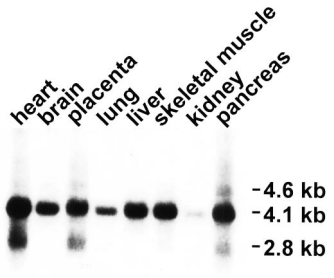

Figure 4. Tissue-specific expression of human tfcD gene. A Northern blot of RNA isolated from different human tissues (10 μg/lane) was hybridized with a human tfcD cDNA probe. The probe detects tissue-specific mRNA species of 4.6, 4.1, and 2.8 kb.

erodimers, suggesting a role as a microtubule destabilizing protein.[23] This is supported by overexpression of tfcD in transfected HeLa cells resulting in a progressive loss of microtubules.[23,24] Therefore, downregulation of tfcD expression in response to arterial laminar shear stress would probably increase the number of microtubules, resulting in stabilization of the microtubular network.

We identified in the human tfcD protein several interesting structural features. The existence of 2 RGD sites usually found in extracellular matrix proteins suggests a role of tfcD in cell adhesion.[25] From the 9 potential myristoylation sites detected by tfcD sequence analysis, most probably only 1 N-terminal site exists in vivo. Furthermore, the putative phosphorylation sites for cAMP- and cGMP-dependent protein kinases, tyrosine kinase, protein kinase C, and casein kinase II represent potential targets for regulation of tfcD activity. The functional importance of each of these structural features must be confirmed in further studies.

A critical role of microtubules in shear stress–dependent reorganization of the cytoskeleton was previously described.[3] Disruption of microtubular network and inhibition of tyrosine kinase activity blocked the shear-induced alignment of cell shape and actin stress fibers. These data suggest a dynamic interaction between the microtubular and actin fiber network in response to mechanical forces.[26] The role of microtubules in this tensegrity model is a stabilization of cytoplasm and

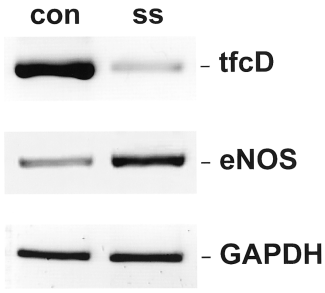

Figure 5. Nuclear run-on assays using a new RT-PCR–based approach performed with nuclei isolated from HUVEC with (ss) or without (con) application of arterial levels of laminar shear stress. tfcD mRNA expression is downregulated by arterial levels of shear stress at the transcriptional level in HUVECs. In contrast, eNOS mRNA is induced in the same nuclei, whereas GAPDH transcription is not affected. Results shown are representative of 3 independent experiments.

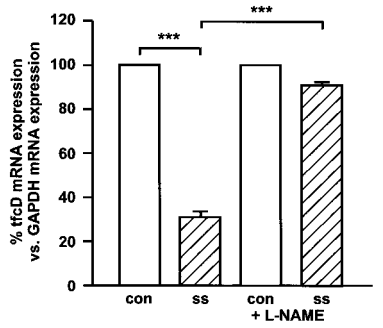

Figure 6. NO-dependent downregulation of tfcD mRNA by arterial shear stress. HUVECs were cultured under static conditions (con, white bars) or exposed to long-term arterial laminar shear stress (ss, dashed bars). Effect of NO synthase inhibition (L-NAME, 400 μmol/L) on downregulation of tfcD mRNA by shear stress was tested. Total RNA was harvested and analyzed by Northern blotting with tfcD and GAPDH cDNA probes as described in Figure 2. The tfcD mRNA expression is normalized vs GAPDH mRNA expression and indicated as percentage of static internal control incubated with or without L-NAME. ($n \geq 4$ each, $^{***}P<0.001$ vs control and ss+L-NAME).

nucleus against lateral compression. Shear stress of low degree (eg, venous shear stress) might induce only minor changes in the microtubular network, resulting in a more flexible cytoskeleton, compared with higher degrees of arterial shear stress with development of actin stress fibers and reorganization of microtubular network, resulting in increased mechanical stiffness. The key role of tyrosine kinases in shear stress–induced reorganization of cytoskeleton[3] could

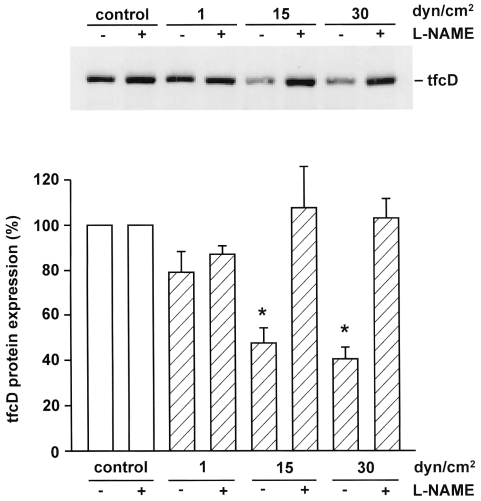

Figure 7. tfcD protein is downregulated by arterial shear stress via a NO-mediated mechanism. HUVECs were cultured under static conditions or exposed for 24 hours to laminar venous (1 dyne/cm^2) or 2 different levels of arterial shear stress (15 or 30 dyne/cm^2) in the absence (−) or presence (+) of the NO synthase inhibitor L-NAME. Subsequently, tfcD protein was quantified by Western blot analysis in these cells. Arterial shear stress resulted in NO-dependent downregulation of tfcD protein in HUVECs. Results shown are representative of 5 independent experiments. In the corresponding bar graph, tfcD protein expression is indicated as percentage of expression under static culture conditions (static condition, white bar; shear stress, dashed bars). $^{*}P<0.05$ vs control without shear stress and corresponding shear stress sample with L-NAME.

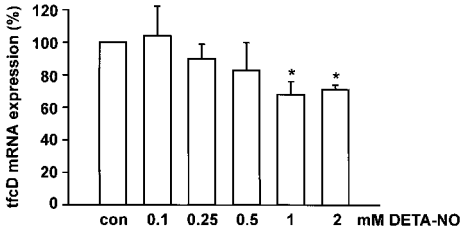

Figure 8. Downregulation of tfcD mRNA by NO. HUVECs were incubated under static conditions with different concentrations of the NO donor DETA-NO for 24 hours. RNA from these cells was isolated, and Northern blot analysis for detection of tfcD mRNA was performed as described in Figure 2. At DETA-NO concentrations higher than 1 mmol/L, tfcD mRNA is significantly downregulated compared with control. The normalized tfcD mRNA expression is expressed as percentage of control without DETA-NO (con) (n=4, *$P<0.05$ vs control).

involve modulation of tfcD activity by phosphorylation of the evolutionarily conserved tyrosine kinase site.

Further studies were aimed at understanding the molecular mechanism underlying this downregulation of tfcD by arterial laminar shear stress. Because application of arterial laminar shear stress was found to induce the endothelial isoform of NO synthase and NO formation as an atheroprotective mechanism,[8–10] we analyzed the effect of NO synthase inhibition on shear stress–dependent downregulation of tfcD mRNA. We also studied the effect of the NO donor DETA-NO on tfcD expression. Our data provide evidence for a NO-dependent downregulation of tfcD. The downregulation of tfcD mRNA by shear stress was much more marked than the effect shown in response to the exogenous NO donor DETA-NO. One possible explanation for this difference could be the need of a long-lasting NO release to get a sustained downregulation of tfcD mRNA. Even while DETA-NO is considered as a compound mediating a long-lasting NO release, the 24-hour incubation period in the cell culture medium containing 5% dextran could decrease the NO level. In contrast, high laminar shear stress is considered as a strong physiological stimulus causing a sustained upregulation of eNOS expression and NO release.[10] This could explain the lower level of downregulation of tfcD mRNA by NO donor DETA-NO, compared with high laminar shear stress. Furthermore, shear stress elicits the activation of other signaling pathways and the generation of additional endothelial autacoids that affect gene regulation without the involvement of NO. NO can affect expression of other genes via cGMP by activation or deactivation of transcription factors.[27] Disruption of microtubular network attenuates flow-dependent NO release in perfused vessels.[28] Our data suggest an additional signal-transduction pathway leading to the opposite direction with NO-mediated regulation of microtubular turnover through β-tubulin folding.

In summary, our data show a NO-dependent downregulation of the tfcD gene by arterial levels of laminar shear stress in human endothelial cells. The tfcD gene might be involved in modulation of endothelial microtubule dynamics in response to shear stress. The higher degree of shear stress in arterial vessels reduces the expression of the microtubule-destabilizing protein tfcD in endothelial cells and makes the cytoskeleton more rigid than the more flexible cytoskeleton in venous endothelial cells. This change in microtubule dynamics seems to be mediated by NO. Therefore, our data suggest a new role of NO as a signaling molecule that transduces mechanical forces into functional changes of the microtubular network in human endothelial cells.

Acknowledgments

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References

1. Asakura T, Karino T. Flow patterns and spatial distribution of atherosclerotic lesions in human coronary arteries. *Circ Res*. 1990;66:1045–1066.
2. Traub O, Berk BC. Laminar shear stress: mechanisms by which endothelial cells transduce an atheroprotective force. *Arterioscler Thromb Vasc Biol*. 1998;18:677–685.
3. Malek AM, Izumo S. Mechanism of endothelial cell shape change and cytoskeletal remodeling in response to fluid shear stress. *J Cell Sci*. 1996;109:713–726.
4. Davies PF, Barbee KA, Volin MV, Robotewskyj A, Chen J, Joseph L, Griem ML, Wernick MN, Jacobs E, Polacek DC, dePaola N, Barakat AI. Spatial relationships in early signaling events of flow-mediated endothelial mechanotransduction. *Annu Rev Physiol*. 1997;59:527–549.
5. Wong AJ, Pollard TD, Herman IM. Actin filament stress fibers in vascular endothelial cells in vivo. *Science*. 1983;219:867–869.
6. Hintze TH, Vatner SF. Reactive dilation of large coronary arteries in conscious dogs. *Circ Res*. 1984;54:50–57.
7. Holtz J, Forstermann U, Pohl U, Giesler M, Bassenge E. Flow-dependent, endothelium-mediated dilation of epicardial coronary arteries in conscious dogs: effects of cyclooxygenase inhibition. *J Cardiovasc Pharmacol*. 1984;6:1161–1169.
8. Nishida K, Harrison DG, Navas JP, Fisher AA, Dockery SP, Uematsu M, Nerem RM, Alexander RW, Murphy TJ. Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase. *J Clin Invest*. 1992;90:2092–2096.
9. Topper JN, Cai J, Falb D, Gimbrone MA Jr. Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. *Proc Natl Acad Sci U S A*. 1996;93:10417–10422.
10. Busse R, Fleming I. Pulsatile stretch and shear stress: physical stimuli determining the production of endothelium-derived relaxing factors. *J Vasc Res*. 1998;35:73–84.
11. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins: identification by morphologic and immunologic criteria. *J Clin Invest*. 1973;52:2745–2756.
12. Morawietz H, Rueckschloss U, Niemann B, Duerrschmidt N, Galle J, Hakim K, Zerkowski HR, Sawamura T, Holtz J. Angiotensin II induces LOX-1, the human endothelial receptor for oxidized low-density lipoprotein. *Circulation*. 1999;100:899–902.
13. Sdougos HP, Bussolari SR, Dewey CFJ. Secondary flow and turbulence in a cone-plate device. *J Fluid Mech*. 1984;138:379–404.
14. Morawietz H, Talanow R, Szibor M, Rueckschloss U, Schubert A, Bartling B, Darmer D, Holtz J. Regulation of the endothelin system by shear stress in human endothelial cells. *J Physiol (Lond)*. 2000;525:761–770.
15. Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry*. 1979;18:5294–5299.

16. Morawietz H, Ma YH, Vives F, Wilson E, Sukhatme VP, Holtz J, Ives HE. Rapid induction and translocation of Egr-1 in response to mechanical strain in vascular smooth muscle cells. *Circ Res*. 1999;84:678–687.
17. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol*. 1990;215:403–410.
18. Krzesz R, Wagner AH, Cattaruzza M, Hecker M. Cytokine-inducible CD40 gene expression in vascular smooth muscle cells is mediated by nuclear factor κB and signal transducer and activation of transcription-1. *FEBS Lett*. 1999;453:191–196.
19. Tian G, Huang Y, Rommelaere H, Vandekerckhove J, Ampe C, Cowan NJ. Pathway leading to correctly folded β-tubulin. *Cell*. 1996;86: 287–296.
20. Tian G, Bhamidipati A, Cowan NJ, Lewis SA. Tubulin folding cofactors as GTPase-activating proteins: GTP hydrolysis and the assembly of the α/β-tubulin heterodimer. *J Biol Chem*. 1999;274:24054–24058.
21. Cowan NJ, Lewis SA. A chaperone with a hydrophilic surface. *Nat Struct Biol*. 1999;6:990–991.
22. Lewis SA, Tian G, Vainberg IE, Cowan NJ. Chaperonin-mediated folding of actin and tubulin. *J Cell Biol*. 1996;132:1–4.
23. Martin L, Fanarraga ML, Aloria K, Zabala JC. Tubulin folding cofactor D is a microtubule destabilizing protein. *FEBS Lett*. 2000;470:93–95.
24. Bhamidipati A, Lewis SA, Cowan NJ. ADP ribosylation factor-like protein 2 (Arl2) regulates the interaction of tubulin-folding cofactor D with native tubulin. *J Cell Biol*. 2000;149:1087–1096.
25. Ruoslahti E. RGD and other recognition sequences for integrins. *Annu Rev Cell Dev Biol*. 1996;12:697–715.
26. Ingber DE. Tensegrity: the architectural basis of cellular mechanotransduction. *Annu Rev Physiol*. 1997;59:575–599.
27. Forstermann U, Boissel JP, Kleinert H. Expressional control of the "constitutive" isoforms of nitric oxide synthase (NOS I and NOS III). *FASEB J*. 1998;12:773–790.
28. Hutcheson IR, Griffith TM. Mechanotransduction through the endothelial cytoskeleton: mediation of flow- but not agonist-induced EDRF release. *Br J Pharmacol*. 1996;118:720–726.

Online-Only Supplementary Information - MS 1368/R1:

Shear stress-dependent regulation of the human β-tubulin folding cofactor D gene

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Material and Methods

Cell culture and application of shear stress

All cell culture reagents and chemicals were purchased from Sigma Chemicals (St. Louis, MO) if not indicated otherwise. Human umbilical vein endothelial cells (HUVEC) were isolated using collagenase IV.[1] In order to minimize variations of primary cultures, each day the isolated HUVEC were pooled, subsequently separated and grown in medium M199 with 1.25 mg/mL sodium bicarbonate, 100 µg/mL L-glutamine (Life Technologies), supplemented with 20% calf serum, 15 mmol/L HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin, 250 ng/mL fungizone (Life Technologies), and 16.7 ng/mL endothelial cell growth supplement (C. C. Pro, Neustadt, Germany).[2] Cells were cultured on 94 x 16 mm tissue culture dishes (Greiner Corp., Solingen, Germany) (approximately 1 x 10^6 cells/dish) and subjected to laminar shear stress one day after reaching confluence in a cone-and-plate viscometer[3] with minor modifications as described.[4] The cone-and-plate viscometer operates in a linear range at any desired rotational speed to achieve shear stress levels of up to 50 dyn/cm^2. Increasing levels of shear stress were applied by increasing rotational speed. In order to avoid direct contact of the rotating cone with the edge of the tissue culture dish, a circular area of 91% of

the cultured cells was exposed to the indicated amount of laminar shear stress. Laminar shear stress of 1 dyn/cm^2 (1 dyn/cm^2 = 0.1 N/m^2, venous or low shear stress), 15 or 30 dyn/cm^2 (15 dyn/cm^2 = 1.5 N/m^2, 30 dyn/cm^2 = 3 N/m^2; arterial or high shear stress) was applied in a humidified environment with 5% CO_2 at 37°C. In order to keep the cell culture medium volume constant and to avoid a spill-over of the medium even at high rotational speed, for application of arterial levels of shear stress 5% dextran (MW 71.400) was added to the cell culture medium to increase the viscosity of the medium 2.95-fold from 0.007 dyn · s/cm^2 to 0.02065 dyn · s/cm^2. In these experiments, each cell culture dish was accompanied by 2 controls from the same HUVEC preparation incubated under static conditions with cell culture medium supplemented with or without 5% dextran for 24 h. Dextran had no effect on β-tubulin folding cofactor D (tfcD) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression in this study (Figure 1 Online). Application of laminar shear stress using the cone-and-plate viscometer did not increase the temperature of the cell culture medium (Figure 2 Online). In order to balance evaporation of cell culture medium during the 24 h application of shear stress on cells in an open cell culture dish, medium was supplemented at regular intervals with sterile distilled water. The achievement of equal degrees of shear stress at lower rotational speed by using additional dextran has been shown to give equal results[5] and did not affect cell viability, detachment or increased release of lactate dehydrogenase (LDH) into the medium as an indicator of cell integrity (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega, Madison, WI) (Figure 3 Online). The flow conditions used in our study were laminar, because the parameter R ($r^2\omega\alpha^2/12\nu$) described by Sdougos et al.[3] was in each case smaller than 4 (R_{1dyn/cm^2}: 0.006; R_{15dyn/cm^2}: 0.03; R_{30dyn/cm^2}: 0.06).

RNA Isolation and RAP-PCR

Total RNA from endothelial cells was isolated by guanidinium thiocyanate/cesium chloride centrifugation.[6] The mRNA was then obtained using an mRNA Purification Kit (Pharmacia).

RNA arbitrarily primed-polymerase chain reaction (RAP-PCR) was used to identify differentially expressed transcripts (RAP-PCR Kit, Stratagene). First, 100 ng mRNA from HUVEC exposed to shear stress (1 dyn/cm^2 or 15 dyn/cm^2, 24 h) were reverse transcribed into cDNA with one of five designed 18-base-primers (50% GC content). Second, these reverse transcribed cDNA species were amplified using this primer, one of the four additional primers, and [α-^{32}P]dATP (Amersham). The RAP-PCR fragments were subsequently separated on 4% polyacrylamide gels containing 7 mol/L urea by electrophoresis, dried and exposed at room temperature to Kodak BIOMAX MS film. Differentially expressed RAP-PCR fragments were excised from the gel, incubated in TE buffer for 1 h at 60°C followed by incubation at 4°C overnight. Finally, an aliquot from this eluate was used for PCR reamplification with the same primers.

Northern blot analysis

Total cellular RNA (10 μg) from HUVEC was denatured with glyoxal/dimethyl sulfoxide, separated on recirculating 1.2% agarose gels in 3-(*N*-morpholino)propanesulfonic acid buffer, transferred to nylon membranes (Stratagene) and fixed by UV crosslinking. The human multiple tissue Northern blot was purchased from Clontech (Palo Alto, CA). RAP-PCR fragments and cDNA clones were labeled using the Oligolabelling Kit (Pharmacia) and [α-^{32}P]dCTP (Amersham), hybridized with membranes in hybridization solution at 65°C for 16 h, and washed and exposed at -80°C to Kodak BIOMAX MS films. Subsequently, membranes

were stripped in 0.1xSSC, 0.1% SDS at 80°C, and hybridizied with a human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe as previously described.[7] The GAPDH gene was used as a control, because the expression of this gene was not affected by laminar shear stress (Figure 4 Online).

cDNA cloning and DNA sequence analysis

The reamplified RAP-PCR fragments were cloned into the pCR-Script Amp SK(+) plasmid (pCR-Script Amp SK(+) Cloning Kit, Stratagene). Single RAP-PCR clones were tested for shear stress-dependent regulation by Northern blot analysis. RAP-PCR fragments showing an identical regulation by shear stress were used to screen $1x10^6$ plaque-forming units of an oligo (dT) and randomly primed human heart-Lambda ZAP II-cDNA library (Stratagene).

The DNA sequence of cDNA clones was determined by cycle sequencing with ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) on an automated ABI PRISM 373A DNA Sequencer (ABI/Perkin Elmer). DNA sequence was analyzed using Gene Runner software (Hastings Software, Inc.). The DNA and deduced protein sequence was analyzed by data base searches of GenBank.[8] Alignment of amino acid sequences was done according to Dayhoff.[9]

Nuclear run-on assays

Nuclear run-on experiments were adapted for HUVEC from a protocol described previously.[10] In brief, HUVEC incubated under static conditions or exposed to arterial levels of shear stress were harvested and incubated for 10 min in 2 volumes of lysis buffer (5 mmol/L Hepes, 1 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol (DTT), 20% glycerol, 0.05%

Triton X-100; pH 7.4). The volume was subsequently adjusted to 2 mL with washing buffer (20 mmol/L Tris, 140 mmol/L KCl, 10 mmol/L $MgCl_2$, 1 mmol/L DTT, protease inhibitor mix; pH 7.4). Cells were lyzed in a cell cracker device,[11] centrifuged for 10 s at 300×*g*, and the resulting supernatant for additional 5 min at 1,650×*g*. The raw nuclear pellet was washed three times with washing buffer and collected by centrifugation as described. Final nuclear pellets were suspended in 4 volumes of reaction buffer (2 mmol/L each CTP, GTP and UTP, 3 mmol/L ATP, 20 U ml^{-1} RNAsin (Fermentas, Vilnius, Lithuania), 8.5 mmol/L creatine phosphate and 0.1 mg/ml creatine kinase (Boehringer, Mannheim, Germany) in washing buffer. Half of nuclei were immediately lyzed in 4 volumes of guanidinium thiocyanate (GTC) buffer (control) and the other half incubated at 30°C for 30 min and subsequently lyzed in GTC buffer. RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis were performed using standard protocols.[12] RT-PCR fragments from control nuclei lyzed immediately after isolation were usually not detectable or less abundant (max. 20% of control).

Inhibitor and NO donor studies

HUVEC were cultured under static conditions, or exposed to laminar shear stress for 24 h (after preincubation of 1 h) with or without the nitric oxide (NO) synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 1 or 400 μM). In these experiments, matched controls from the same cell preparation were supplemented with dextran and inhibitor as well and incubated for the same period of time without application of shear stress.

In order to analyze the effect of NO on tfcD expression, static cultures of HUVEC supplemented with dextran medium were incubated with the NO donor DETA-NO (0.1-2 mmol/L) for 24 h, and subsequently studied by Northern blot analysis.

Production of antibodies directed against SSD-1 peptide

One strongly immunogenic peptide of shear stress down-regulated gene-1 (SSD-1) protein was selected using Gene Runner software (Hastings Software, Inc.). A 14-mer immunogenic peptide (VKKEIKNSKDIQKL, SSD-1 residues 1075-1088) was synthesized and conjugated with an additional N-terminal cysteine residue to Keyhole Limpet Hemocyanin (KLH), and used for immunization of rabbits (Eurogentec, Berlin, Germany). Specificity of pre-immune and SSD-1 antiserum was tested in slot blot and Western blot analysis.

Protein isolation and Western blot analysis

After static culture or application of shear stress, cells were harvested in PBS, lyzed in 0.5% SDS/PBS, boiled and centrifuged for 10 min at 14,000 rpm and 4°C. The protein concentration was determined with BCA Protein Assay Reagent (Pierce Corp., Rockford, IL). Proteins (10 μg/lane) were separated by SDS-PAGE (7.5%) and transferred to Hybond ECL nitrocellulose membranes (Amersham). Membranes were incubated with SSD-1 antiserum, and secondary horseradish peroxidase-linked rabbit Ig, and visualized with the ECL Western blotting detection reagent (Amersham).

Statistics

In experiments with dimensionless quantities, band densities from multiple similar experiments were combined by calculation of the fold increase or decrease vs. control under each experimental condition. Data are given as mean±S.E.M. ($n \geq 3$ in all cases). Statistical analysis was performed with the ANOVA procedure followed by Bonferroni's *t* test (multiple comparison) or Student's *t* test (SigmaStat software, Jandel Corp). Differences were taken as statistically significant at $P<0.05$.

Results

Shear stress down-regulated gene-1 encodes human β-tubulin folding cofactor D

The shear stress down-regulated gene-1 (SSD-1) amino acid sequence revealed 80.4% identity and 90.9% homology to the recently cloned bovine β-tubulin folding cofactor D (tfcD).[13] The alignment of the amino acid sequences of human SSD-1 encoding tfcD with bovine tfcD is shown in Figure 5 Online.

References

1. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest*. 1973;52:2745-2756.
2. Morawietz H, Rueckschloss U, Niemann B, Duerrschmidt N, Galle J, Hakim K, Zerkowski HR, Sawamura T, Holtz J. Angiotensin II induces LOX-1, the human endothelial receptor for oxidized low-density lipoprotein. *Circulation*. 1999;100:899-902.
3. Sdougos HP, Bussolari SR, Dewey CFJ. Secondary flow and turbulence in a cone-plate device. *J Fluid Mech*. 1984;138:379-404.
4. Morawietz H, Talanow R, Szibor M, Rueckschloss U, Schubert A, Bartling B, Darmer D, Holtz J. Regulation of the endothelin system by shear stress in human endothelial cells. *J Physiol (Lond)*. 2000;525:761-770.

5. Malek A, Izumo S. Physiological fluid shear stress causes downregulation of endothelin-1 mRNA in bovine aortic endothelium. *Am J Physiol*. 1992;263:C389-C396.

6. Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry*. 1979;18:5294-5299.

7. Morawietz H, Ma YH, Vives F, Wilson E, Sukhatme VP, Holtz J, Ives HE. Rapid induction and translocation of Egr-1 in response to mechanical strain in vascular smooth muscle cells. *Circ Res*. 1999;84:678-687.

8. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol*. 1990;215:403-410.

9. Dayhoff MO. *Atlas of protein sequence and structure 5, suppl. 3*. Washington DC; 1978.

10. Krzesz R, Wagner AH, Cattaruzza M, Hecker M. Cytokine-inducible CD40 gene expression in vascular smooth muscle cells is mediated by nuclear factor kappaB and signal transducer and activation of transcription-1. *FEBS Lett*. 1999;453:191-196.

11. Balch WE, Dunphy WG, Braell WA, Rothman JE. Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylglucosamine. *Cell*. 1984;39:405-416.

12. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. *Anal Biochem*. 1987;162:156-159.

13. Tian G, Huang Y, Rommelaere H, Vandekerckhove J, Ampe C, Cowan NJ. Pathway leading to correctly folded beta-tubulin. *Cell*. 1996;86:287-296.

Figure Legends

Figure 1 Online. Effect of dextran on β-tubulin folding cofactor D or glyceraldehyde 3-phosphate dehydrogenase mRNA expression in HUVEC. The mRNA expression of β-tubulin folding cofactor D (tfcD) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was determined in RNA from HUVEC after incubation with cell culture medium with or without 5% dextran for 24 h by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) in the linear range using the light cycler system. Identical results were obtained by Northern blot analysis. Equal amounts of RNA (determined spectrophotometrically) were analyzed in these experiments. The tfcD and GAPDH mRNA expression was normalized by real-time RT-PCR using 18SrRNA-specific primers, or by scanning the amount of 18SrRNA in ethidium bromide stained RNA agarose gels for equal loading in Northern analyses, when appropriate. Dextran did not affect tfcD mRNA expression (P=0.284) or GAPDH mRNA expression (P=0.586).

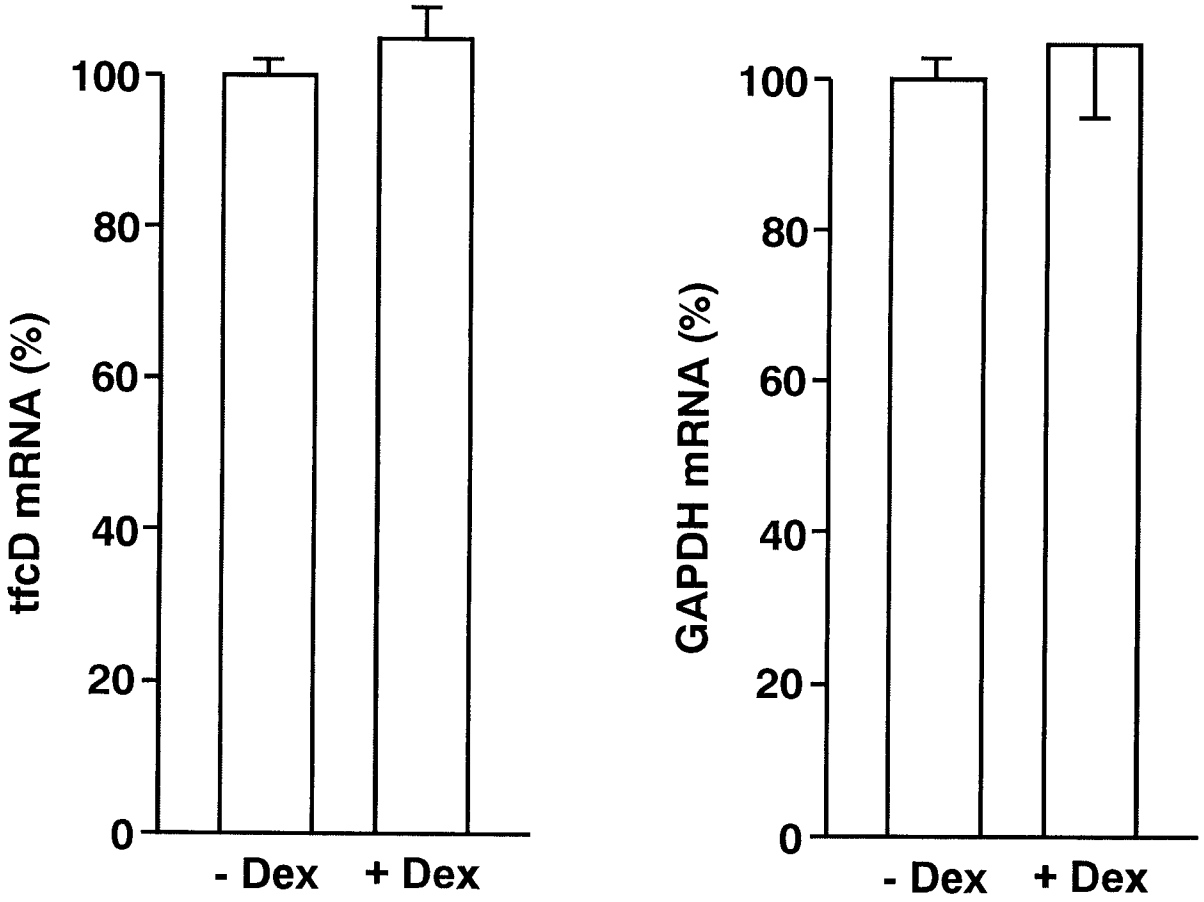
Figure 2 Online. Application of laminar shear stress using the cone-and-plate viscometer does not increase the temperature of the cell culture medium. The temperature was determined using a microthermometer allowing the measurement of the medium temperature in the closed incubator. Application of laminar shear stress for 24 h of 1, 15, or 30 dyn/cm^2 had no effect on the temperature of the cell culture medium (P=0.648).

Figure 3 Online. Application of laminar shear stress does not increase lactate dehydrogenase release of human endothelial cells. The lactate dehydrogenase (LDH) release into the cell culture medium as an indicator of cell integrity was measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI). Application of low (1 dyn/cm^2) or high (30 dyn/cm^2) levels of laminar shear stress on primary cultures of human umbilical vein endothelial cells using the cone-and-plate apparatus did not increase LDH release into the medium (P=0.56).

Figure 4 Online. The glyceraldehyde 3-phosphate dehydrogenase mRNA expression is not affected by laminar shear stress. Human umbilical vein endothelial cells (HUVEC) were exposed to different levels of long-term laminar shear stress (24 h). In each experiment an internal control without application of shear stress was included. The mRNA expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was determined in RNA from HUVEC by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) in the linear range using the light cycler system. Equal amounts of RNA (determined spectrophotometrically) were analyzed in each experiments. The GAPDH mRNA expression was normalized to 18SrRNA, amplified by real-time RT-PCR from the same RT reaction using 18SrRNA-specific primers, and expressed as % of control. Shear stress did not affect GAPDH expression (P=0.844).

Figure 5 Online. Alignment of amino acid sequences of human shear stress down-regulated gene-1 (SSD-1) with bovine β-tubulin folding cofactor D (tfcD). SSD-1 encodes the human homologue of bovine tfcD.[13] Identical amino acids are indicated by vertical bars, similar amino acids, as defined by the rules of Dayhoff,[9] are indicated by dots between the sequences.

Figures Online



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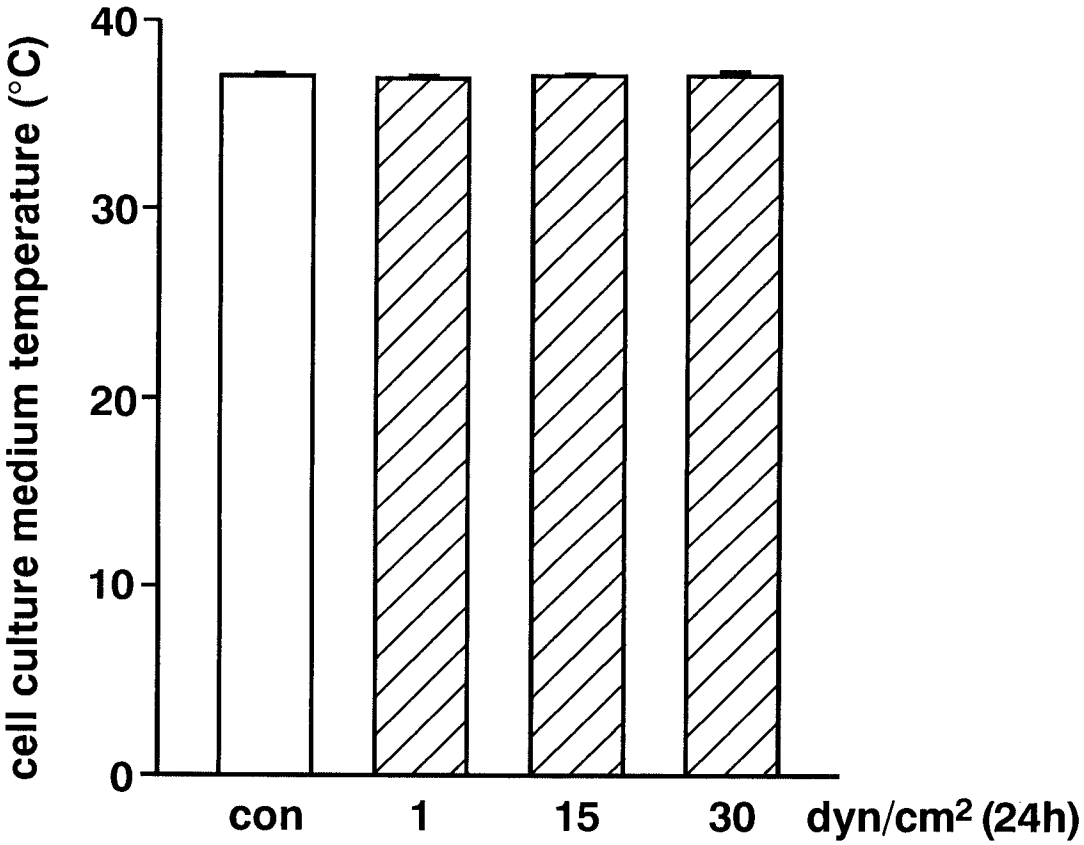

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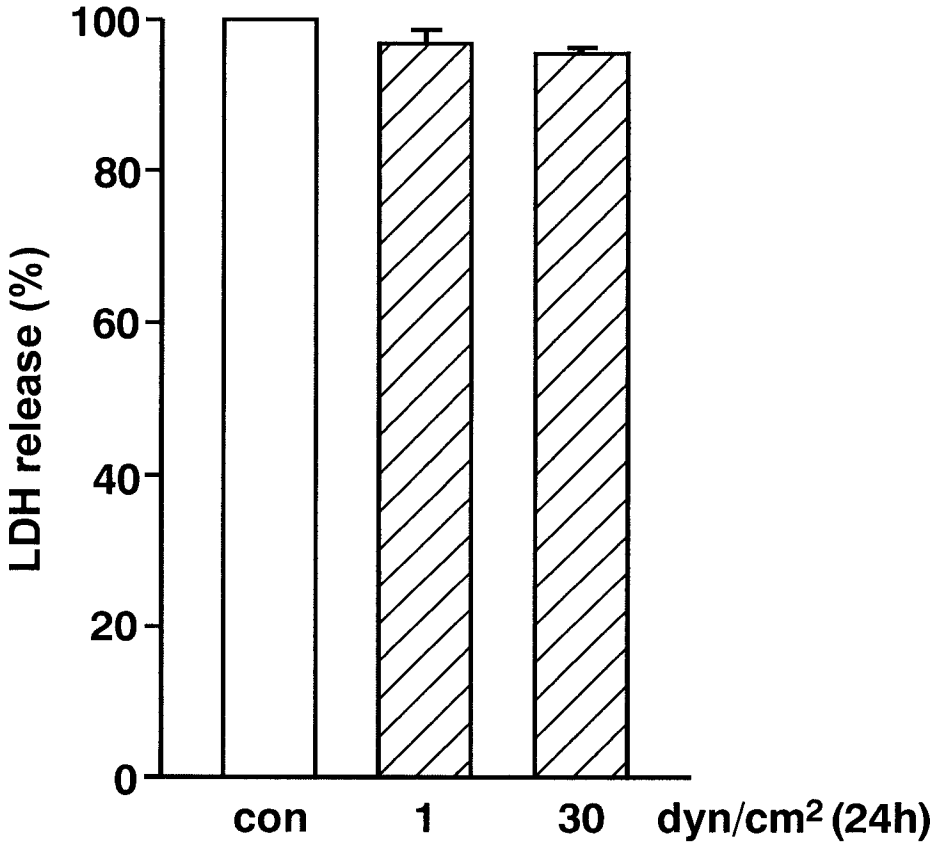

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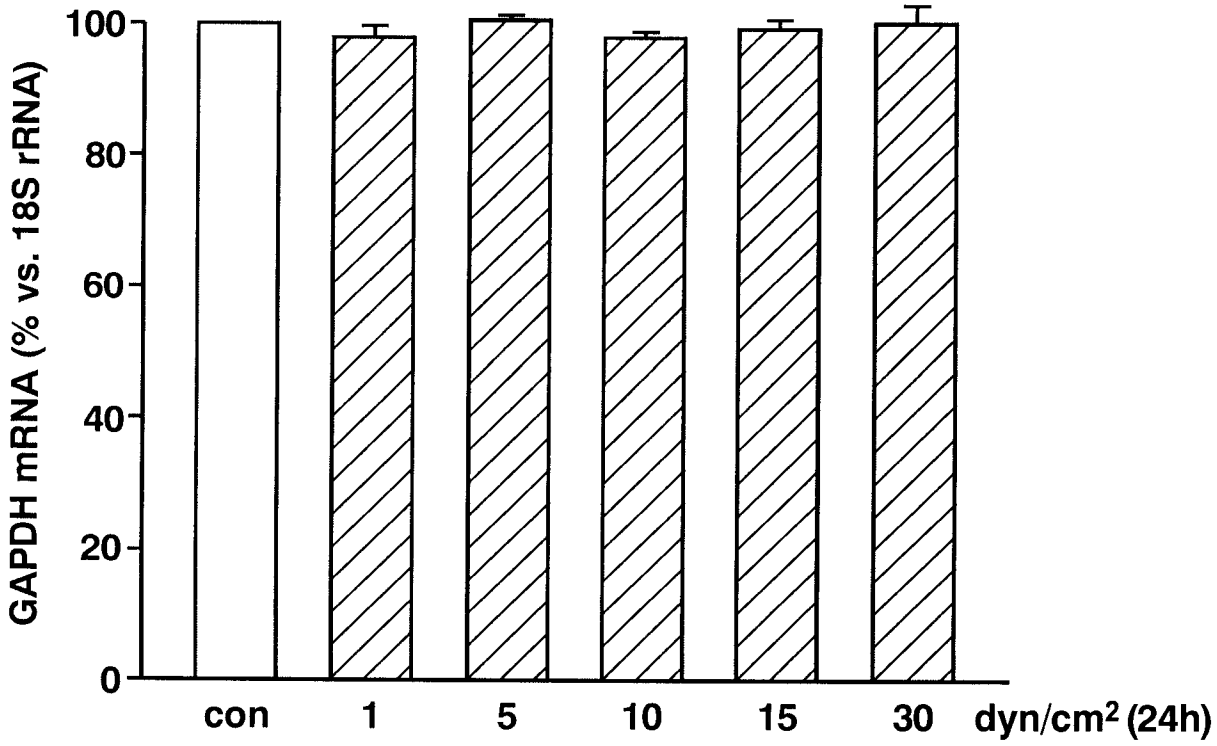

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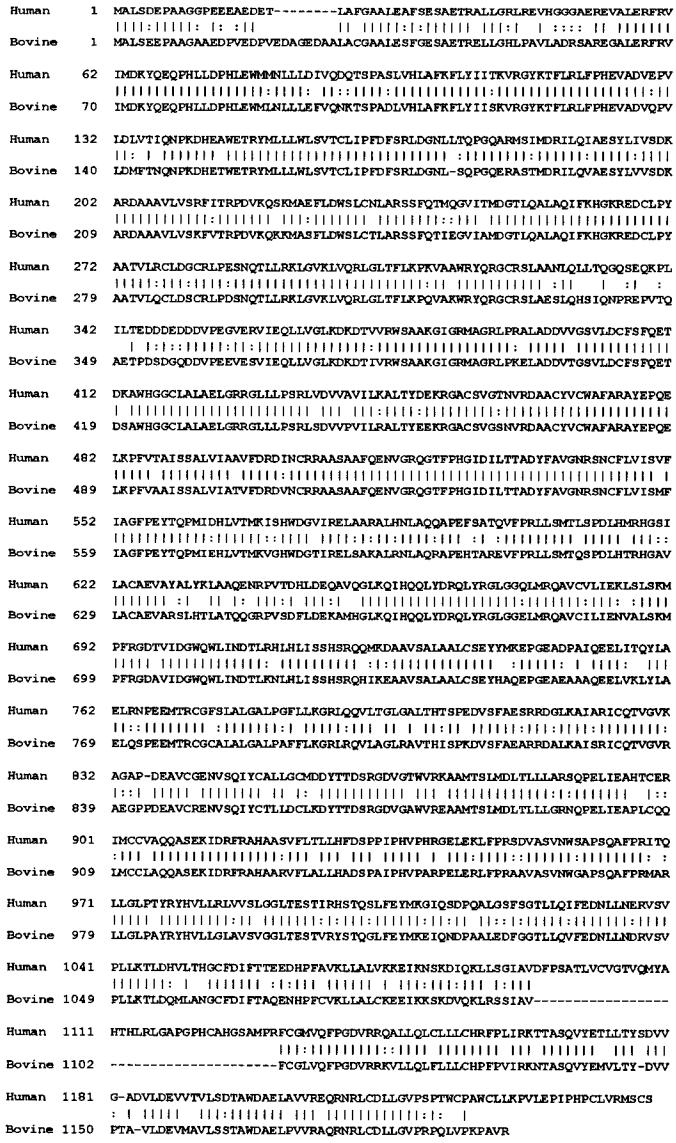

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