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Regulation of Foxo-1 and the angiopoietin-2/Tie2 system by shear stress

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Abstract Transcription factor Foxo-1 can be inactivated via Akt-mediated phosphorylation. Since shear stress activates Akt, we determined whether Foxo-1 and the Foxo-1-dependent, angiogenesis-related Ang-2/Tie2-system are influenced by shear stress in endothelial cells. Expression of Foxo-1 and its target genes p27Kip1 and Ang-2 was decreased under shear stress (6 dyn/cm^2, 24 h), nuclear exclusion of Foxo-1 by phosphorylation increased. eNOS and Tie2 were upregulated. No effects on Ang-1 expression were detected. In conclusion, Foxo-1 and Ang-2/Tie2 are part of the molecular response to shear stress, which may regulate angiogenesis.

Keywords: Angiogenesis; Shear stress; Endothelium; Foxo-1; Ang-2; Tie2

1. Introduction

Blood vessel growth (angiogenesis) is correlated with changes in hemodynamic forces [1]. Especially wall shear stress, the tensile force tangentially acting on endothelial cells exposed to the flowing blood, is significantly altered prior or at least during the different modes of angiogenesis. In sprouting type of angiogenesis, there is no blood flow in not yet perfused capillary sprouts. Oppositely, splitting type of angiogenesis in skeletal muscle can be initiated by an increase of wall shear stress [2]. However, important aspects of the molecular link between shear stress and its effects on the regulation of angiogenesis are still missing.

A primary regulator of shear/stretch effects of endothelial mechanostimulation is the serine/threonine kinase Akt [3]. Activation of Akt through phosphatidyl inositol 3 kinase (PI3K) leads to phosphorylation and nuclear exclusion of the transcription factor Forkhead box protein O1A (Foxo-1). Accordingly, Foxo-1 dephosphorylation leads to translocation to the nucleus and induces the transcription of target genes such as Fas, BIM, p27 Kip1 that trigger apoptosis [4]. With respect to angiogenesis-related molecules, Foxo-1 induces the expression of angiopoietin-2 (Ang-2) [5], a competitive antagonist of angiopoietin-1 (Ang-1). Ang-1 and its endothelial receptor tyrosine-protein kinase receptor (Tie2) are required for vascular development, maturation, and stability [6]. Albeit Ang-2 also binds to Tie2, it does not activate this receptor under physiological conditions and is consequently associated with blood vessel destabilization and remodelling [6]. Ang-1 inhibits Foxo-1 and thereby the expression of its antagonist Ang-2. On the opposite, this regulation implies a positive feedback loop in which an increase in Ang-2 expression, blocking Ang-1 effects, leads indirectly to activation of Foxo-1 resulting in a further increase in the expression of Ang-2 and other Foxo-1 target genes. Therefore, it was concluded that even relatively small initial changes in Ang-2 levels could have dramatic effects on blood vessel stability [5].

This study was designed to test the hypothesis that the expression of Foxo-1 and its target gene Ang-2 in endothelial cells is regulated by shear stress.

2. Materials and methods

2.1. Isolation and culture of HUVEC and culture of cell line EAhy926

Human umbilical vein endothelial cells (HUVEC) were isolated as described [7]. Culture medium MCDB 131 (500 ml; Biochrom, Berlin, Germany) was supplemented (SupplementPack MV®, PromoCell, Heidelberg, Germany). At the first passage, HUVEC were seeded on Primaria® culture dishes (100 mm diameter; Becton Dickinson, France) and exposed to shear stress one day after confluency. Endothelial like cell line EAhy926 was grown in Dulbeccos MEM (Biochrom, Berlin, Germany) and exposed to shear stress in the same way as described for HUVEC.

2.2. Shear stress

Shear stress was applied on HUVEC monolayers using a cone-and-plate system as described [7]. Briefly, a rotating cone was inserted into a 100-mm Petri dish providing uniform wall shear stress (τ_w) over the entire cross-sectional area according to the formula $\tau_w = (\omega/\alpha)\eta$. The angular velocity was varied to achieve the appropriate amount of constant wall shear stress. The control samples were isolated from the same umbilical vein and cultivated under non-flow conditions for the same time.

2.3. Inhibition of PI-3-kinase

PI3K was inhibited using LY-294002 (Sigma-Aldrich, Taufkirchen, Germany). A stock solution (10 mM) was prepared in dimethylsulfoxide and used at a final concentration of 10 μM. HUVEC were preincubated for 30 min and then exposed to shear stress as described above.

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Abbreviations: Akt, RAC-alpha serine/threonine-protein kinase; Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; eNOS, endothelial NO-synthase; Foxo-1, Forkhead box protein O1A; HUVEC, human umbilical vein endothelial cells; Kip, cyclin-dependent kinase inhibitor p27; PI3K, phosphatidyl inositol 3 kinase; Tie2, tyrosine-protein kinase receptor; VEGF, vascular endothelial growth factor

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2.4. Semi-quantitative RT-PCR

Two microgram of total isolated RNA (RNeasy Mini kit, Qiagen, Hilden, Germany) and 1 μg of oligo-dT-15-primer (Promega, Madison, WI, USA) in a total volume of 15 μl were heated (70 °C, 8 min) and chilled on ice. Two hundred units M-MLV reverse transcriptase (Promega) and 5 μl of 5× RT buffer (Promega) were added to a final volume of 25 μl, incubated (42 °C, 1 h), and the enzyme finally inactivated (70 °C, 10 min). 18 μl of RNAse-free water were added, and aliquots of 4 μl used for PCR. Primer pairs for GAPDH were upstream 5′-ATGTTCCAATATGATTCCACCC-3′ (nucleotide position 3312–3333) and downstream 5′-CTGTAGCCAAATTCGTTGTCATAC-3′ (nucleotide position 4723–4746), product size 832 bp; for Ang-1 upstream 5′-AGCTAGAGAAGCAACTTCTTCAACA-3′ (nucleotide position 824–848) and downstream 5′-CTTCACGATGTTGTATTACAGTCCA-3′ (nucleotide position 1279–1303), product size 480 bp; for Ang-2 upstream 5′-CATGCTCCAGATTAGAGCCTGTAAA-3′ (nucleotide position 1972–1996) and downstream 5′-GTGACAGCAGCGTCTGTAAACTGTC-3′ (nucleotide position 2111–2135), product size 164 bp; for Tie2 upstream 5′-CGTGTGAGAAGGCTTGTGAA-3′ (nucleotide position 897–916) and downstream 5′-GCTTACAATCTGGCCCGTAA-3′ (nucleotide position 1066–1085), product size 189 bp. PCR mix contained 10 mM Tris–HCl, 0.1% (v/v) Triton X-100, 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM dNTP, and 1.0 U *Taq* DNA polymerase (Promega). Primers were added at final concentrations of 200 nM (Ang-2), 1 μM (Ang-1, Tie2), and 300 nM (GAPDH). PCR was started at 94 °C for 60 s followed by 27 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 75 s, and finished at 72 °C for 300 s, optimised to remain in the exponential phase of amplification. GAPDH was amplified using the same samples and PCR mix in separate reaction tubes to normalize the results. Non-template controls and no-RT controls were run for all reactions. PCR products were separated on agarose gels and detected by staining with ethidium bromide. Gels were analysed using ONE-Dscan (Scanalytics, USA).

2.5. Real time-PCR

External standards were produced by RT-PCR as described above using the following primers (1 μM final) for GAPDH: upstream 5′-CGACCACTTTGTCAAGCTCA-3′ (1014–1033) and downstream 5′-AGGGGTCTACATGGCAACTG-3′ (1241–1222), product size 228 bp, Ang-2: upstream 5′-GAAAGAAGCAATATCAGGTCCAGCA-3′ (438–463) and downstream 5′-GCCGTTGAACTTATTTGTGTTCTGC-3′ (1762–1787), product size 1325 bp and for Foxo-1: upstream 5′-TGTCCTACGCCGACCTCATC-3′ (binding position 873–892) and downstream 5′-CTTCTTGGCAGCTCGGCTTC-3′ (binding position 1204–1185), product size 332 bp. PCR was run for 40 cycles, aliquots analysed on agarose gels, PCR products isolated from tubes using MSB Spin PCRapace (Invitek, Berlin, Germany), and diluted to 10^8–10^2 copies/μl. Amplification reactions were performed in a final volume of 20 μl, containing 1 μl of standard dilutions or 4 μl cDNA samples as a template, primers for GAPDH (upstream 5′-CAACGAATTTGGCTACAGCA-3′, downstream 5′-AGGGGAGATTCAGTGTGGTG-3′, binding position 1047–1066, 1216–1197, product size 170 bp), for Ang-2 (upstream 5′-TTGGAACACTCCCTCTCGA-3′, downstream 5′-GGATGATGTGCTTGTCTT-3′, binding position 830–850, 962–983, product size 133 bp) or Foxo-1 (upstream 5′-CACGCTGTCGCAGATCTACG-3′, downstream 5′-GGAGATTCCCGCTCTTGCC-3′, binding position 928–947, 1134–1115, product size 207 bp) at a final concentration of 0.3 μM, and 10 μl Master mix SYBR-Green (QuantiTect SYBR Green PCR Kit, Qiagen). PCR was performed using a Rotor Gene 2000 cycler (LTF, Wasserburg, Germany) at 94 °C for 900 s, followed by 45 cycles of 94 °C for 15 s, 56 °C for 30 s, and 72 °C for 75 s. Amplification products were controlled by melting curves. No-RT- and non-template controls were run for all reactions. The values for Foxo-1 were normalized to the corresponding GAPDH values.

2.6. Transfection of EAhy926 with siRNA

Transfection was done according to the manufacturers protocol (siRNA Transfection Protocol, Dharma *FECT*™ 1, HUVEC; Dharmacon Inc., Chicago, USA) with minor modifications. Briefly, 10 μl siRNA against Foxo-1 (5′-GCAGACAUCUGCAGUUAACTT, 100 μM stock solution, MWG, Munich, Germany), GAPDH (50 μM stock solution, labelled with CY-3, Ambion, Huntingdon, Cambridgeshire, UK) or 20 μl negative control siRNA (50 μM stock solution, #4 from Ambion) were incubated in 490 μl or 480 μl cell culture medium without serum and antibiotics for 5 min at room. In parallel, 15 μl of transfection reagent Dharma *FECT*1 were incubated in 485 μl cell culture medium without serum and antibiotics for 5 min at room temperature. Then, siRNA and the transfection reagent were incubated together for another 20 min at room temperature. Four millilitre complete cell culture medium were added to achieve 5 ml of transfection medium in which subconfluent EAhy926 cells were incubated in Primaria® culture dishes (100 mm diameter; Becton Dickinson, France) for 24 h in humidified atmosphere with 5% CO_2 at 37 °C.

2.7. Protein extraction

HUVEC were cooled on ice, suspended with a cell scraper in 1 ml extraction buffer containing 1% (w/v) Triton X-100, 20 mM sodium-phosphate-buffer (pH 7.8), 150 mM NaCl, 2.5 mM EDTA, 1 mM Na_2VO_4, 50 mM NaF and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.02 μg/μl aprotinin, 0.02 μg/μl leupeptin and 0.02 μg/μl Pepstatin A), and homogenized using a syringe with a needle. Homogenates were incubated on ice (at 4 °C) for 20 min with occasional stirring, centrifuged ($20\,000 \times g$ for 15 min), supernatants transferred into a fresh tube, and protein concentrations determined with Bradford-Kit (Biorad, Munich).

2.8. Antibodies

Antibodies were used detecting Foxo-1 in a 1:1500 dilution, phospho-Foxo-1 in a 1:2000 dilution (Cell Signaling Technology, Beverly, MA, USA, 9462 and 9465, respectively), p27Kip1 and endothelial NO-synthase (eNOS) (BD Biosciences, Erembodegem, Belgium, clones 57 and 3, respectively) in a 1:1000 and 1:4000 dilution, respectively, Ang-2 in a 1:500 dilution, Tie2 in a 1:1000 dilution, and phospho-Tyr in a 1:500 dilution (Santa Cruz Biotechnologies, Heidelberg, Germany, sc-7015, sc-324, and sc-508, respectively), Akt and phospho-Ser-473-Akt (active Akt) both in a 1:1000 dilution (Cell Signaling Technology).

2.9. SDS–PAGE and immunoblotting

Fifty microgram of cell extracts were mixed with Laemmli sample buffer [8] and heated at 95°C for 3 min. After separation of proteins by SDS–PAGE, proteins were transferred to nitrocellulose membranes, which were then blocked with 5% (w/v) fat-free, dry milk powder in washing buffer (0.1% v/v Tween 20 in PBS), blot membranes were incubated with primary antibodies for 2 h at room temperature and subsequently for 45 min with a 1:5000 dilution of peroxidase-conjugated secondary antibody (DAKO, Hamburg, Germany, P0448 and P0260). Unbound primary and secondary antibodies were removed by three incubations (6 min each) in washing buffer. Equal protein loading was confirmed by Ponceau staining. Immunoblots were developed by chemiluminescence as previously described [9] using an ECL detection kit (Perkin Elmer, Boston, MA, USA), visualised by exposing blots to Hyperfilm ECL (Amersham, Freiburg, Germany) for varying times, and scanned for densitometric quantification (Quantity 1D analysis, BioRad, München, Germany).

2.10. Immunoprecipitation

Protein (500 μg) were lysed in extraction buffer and incubated with 1 μg or 2 μg anti-P-Foxo antibody (Cell Signaling, Beverly, MA, USA) or anti-Tie2 antibody (see above) for 2 h at 4 °C. Protein A Sepharose (40 μl) (Sigma-Aldrich, Munich) was added and incubation of samples continued overnight at 4 °C with end-over-end agitation. After centrifugation (10 s at $5000 \times g$) supernatants were aspirated, pellets washed four times with 1 ml of 500 mM NaCl-buffer on ice, resuspended in 20 μl of 1.5-fold Laemmli sample buffer (without DDT), and proteins eluted and denatured by heating samples at 95 °C for 3 min.

2.11. Fluorescence microscopy of Foxo-1

Cells were fixed with methanol at −20 °C for 10 min, washed in PBS with 1% BSA, incubated with anti-Foxo-1 antibody (see above) 1:100 in PBS/BSA for 30 min at room temperature, washed twice in PBS/BSA, followed by an incubation with Cy-3-conjugated goat-anti-rabbit IgG (Jackson Immunoresearch Laboratories, Suffolk, UK) 1:1000 in PBS/BSA for another 30 min, washed again two times with PBS and directly analysed using an Ortholux microscope equipped with a water immersion objective (25/0.60W FLUORES-

ZENZ), and filter set N2.1 for epi-fluorescence (all supplied by Leitz, Wetzlar, Germany). Photos were taken with constant time exposure using a Canon EOS 10D.

2.12. Statistical analysis

"*n*" refers to the number of umbilical cords from which HUVEC had been isolated. Cells from the same umbilical cord were split and either exposed to shear stress or kept under static control conditions. Data are given as mean ± S.D. from at least three independent experiments. Statistical analysis was done using Student's one-tailed *t*-test for paired samples, and statistical significance set at a value of $P \leqslant 0.05$.

3. Results

3.1. Effect of shear stress on Foxo-1

Fig. 1 shows a significant decrease of Foxo-1 mRNA-expression in HUVEC caused by shear stress (6 dyn/cm^2, 24 h). Western blotting showed that the amount of Foxo-1-protein is also significantly lower under shear stress (6 dyn/cm^2, 24 h) than under static conditions (Fig. 3, panel A). Although the difference in the expression of Foxo-1 under static conditions and shear stress became smaller with proceeding time (Fig. 2), it remained statistically significant for at least 24 h. Phosphorylation of Foxo-1 increased under the influence of shear stress (Fig. 3, panel C). Shear stress-dependent activation of HUVEC (6 dyn/cm^2, 60 min) reduced the relative number of cells with Foxo-1 located in the nucleus (Foxo-1 in nucleus plus cytosol and Foxo-1 in nucleus only) from about 97% to 30% (Fig. 5).

3.2. Effect of shear stress on Foxo-1 depending proteins

p27 Kip1 protein level decreased in the same way as Foxo-1 did under shear stress (6 dyn/cm^2, 24 h) as shown in Fig. 3, panel B. Using the same samples, we analysed the total protein amount of eNOS, which significantly rose under dynamic conditions (Fig. 3, panel B).

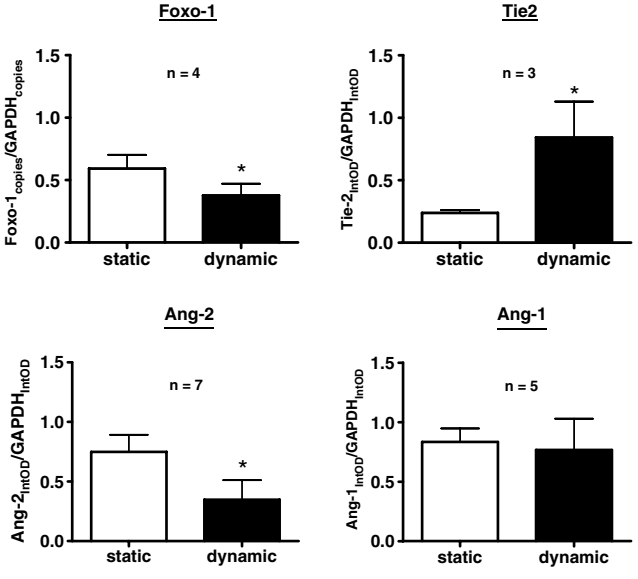

Fig. 1. Changes in mRNA-expression by shear stress. HUVEC were exposed to dynamic (6 dyn/cm^2, 24 h) or kept under static control conditions and analysed for differential expression of mRNA transcripts by RT-PCR. Data are given as means ± S.D. of "*n*" experiments, $^*P \leqslant 0.05$.

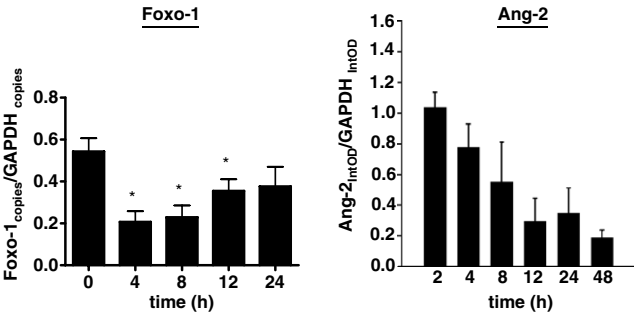

Fig. 2. Time course of Foxo-1- and Ang-2-mRNA-suppression by shear stress. HUVEC were exposed to shear stress (6 dyn/cm^2) for varying times as indicated and analysed by RT-PCR. Data are given as means ± S.D. of three (Foxo-1) and 4 (Ang-2) independent experiments, $^*P \leqslant 0.05$ vs paired static control samples.

3.3. Effect of shear stress on Ang-1, Ang-2, and Tie-2

Expression of Ang-2 decreased under dynamic conditions (6 dyn/cm^2, 24 h) as shown by RT-PCR in Fig. 1. Shear stress-mediated suppression of Ang-2 enhanced with time for at least 48 h (Fig. 2). The total amount of Ang-2 protein became lower in shear stress-exposed HUVEC as shown in Fig. 3 (panel A) for 6 dyn/cm^2 and 24 h. Suppression of Ang-2 increased with the shear force applied with the near complete effect achieved at about 1 dyn/cm^2 (Fig. 7). By contrast, Tie2 was induced under shear stress (6 dyn/cm^2, 24 h) as shown in Fig. 1. The total amount of Tie2 protein increased under shear stress (Fig. 3, panel A). Phosphorylation of Tie2 seemed also to be increased under shear stress (Fig. 3, panel D). Ang-1 was not regulated (Fig. 1).

3.4. Role of Akt in shear stress-dependent regulation of Foxo-1 and Ang-2

Akt is strongly activated through phosphorylation by shear stress (6 dyn/cm^2, 60 min), shown in Fig. 4, panel A. LY-294002, a specific inhibitor of PI3K, reduced shear stress-induced phosphorylation (activation) of Akt almost completely (Fig. 4, panel A). As a consequence, shear stress-induced suppression of Foxo-1 protein was strongly inhibited (Fig. 4, panel B). However, despite inactivation of PI3K, Foxo-1 protein decreased with time if one compares 60 min and 24 h of shear stress acting on the cells (Fig. 4, panel B). Shear stress-induced suppression of Ang-2 was to a small part inhibited by LY-294002 treatment on the mRNA-level (Fig. 4, panel C), suggesting that after 24 h of 6 dyn/cm^2 shear stress about one-third of Ang-2 suppression was due to posttranscriptional inactivation of Foxo-1. In addition, Ang-2 was downregulated on the protein level, although posttranscriptional inactivation of Foxo-1 had been inhibited (Fig. 4, panel C).

3.5. Suppression of Foxo-1-mRNA and Ang-2-mRNA by siRNA against Foxo-1

Since transfection rates of freshly isolated HUVEC were too low, EAhy926 cells were used for experiments with siRNA against Foxo-1. Expression of Foxo-1- and Ang-2-mRNA in these cells was analysed by real time RT-PCR and proved to

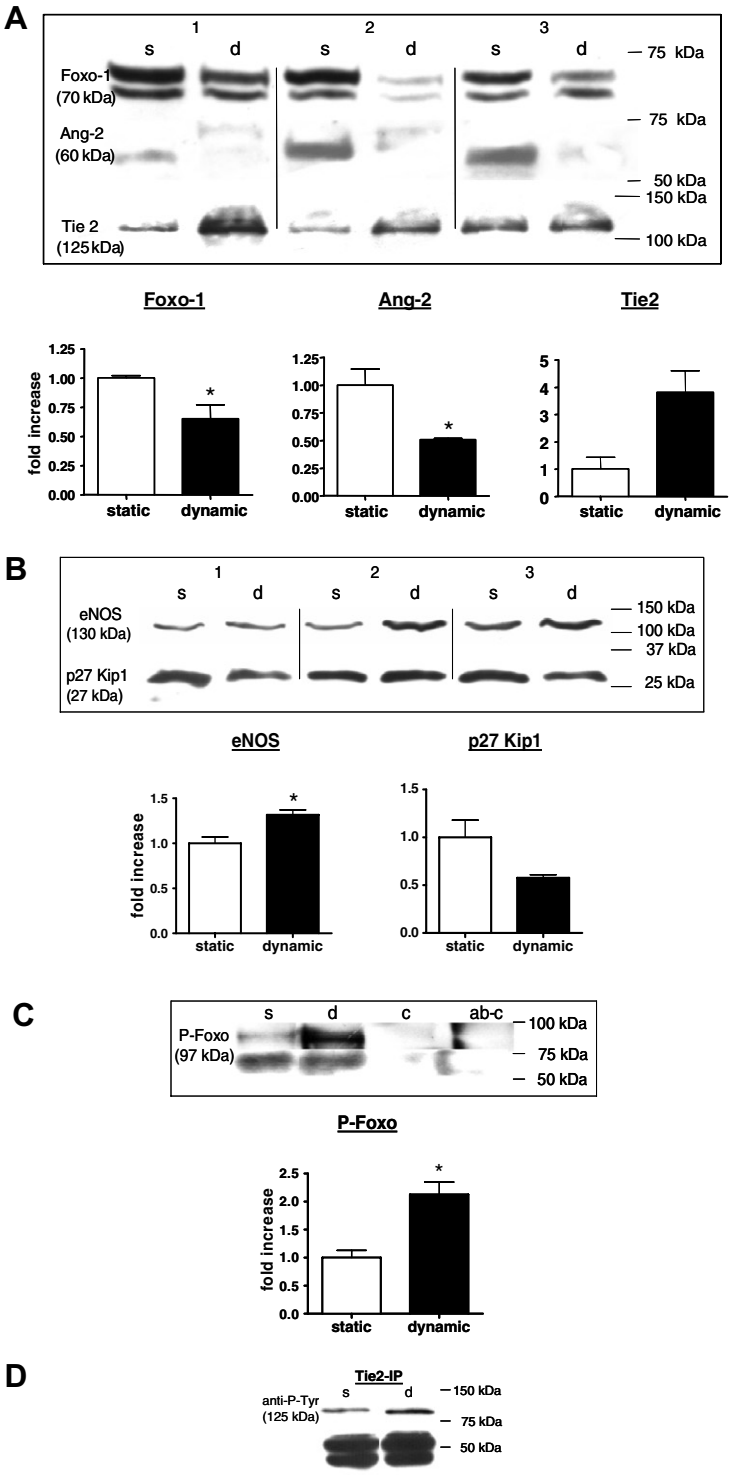

Fig. 3. Changes in protein expression by shear stress. HUVEC were exposed to shear stress (6 dyn/cm^2, 24 h) designated as dynamic conditions (d) or kept under static control conditions (s) and three independent paired samples (1–3) analysed for different protein concentrations by immunoblotting (panels A and B) or immunoprecipitation (panels C and D). Immunoprecipitation of phospho-Foxo is shown with negative control (c), antibody control (ab-c) and IgG heavy chains as a control of the amount of antibodies used for immunoprecipitation. Tie2 was immuno precipitated followed by an anti-P-Tyr-Immuno blot (panel D, $n = 1$). Densitometric data are given as means ± S.D. of three independent experiments, $^*P \leqslant 0.05$.

be somewhat lower compared to HUVEC, but similarly suppressed by shear stress (Fig. 6). siRNA against Foxo-1 suppressed both, expression of Foxo-1- and Ang-2-mRNA during no-flow conditions compared with transfections of negative control siRNA (Fig. 6). Suppression of both mRNA species by siRNA against Foxo-1 was comparable to that achieved by shear stress (6 dyn/cm^2, 24 h) without siRNA (Fig. 6).

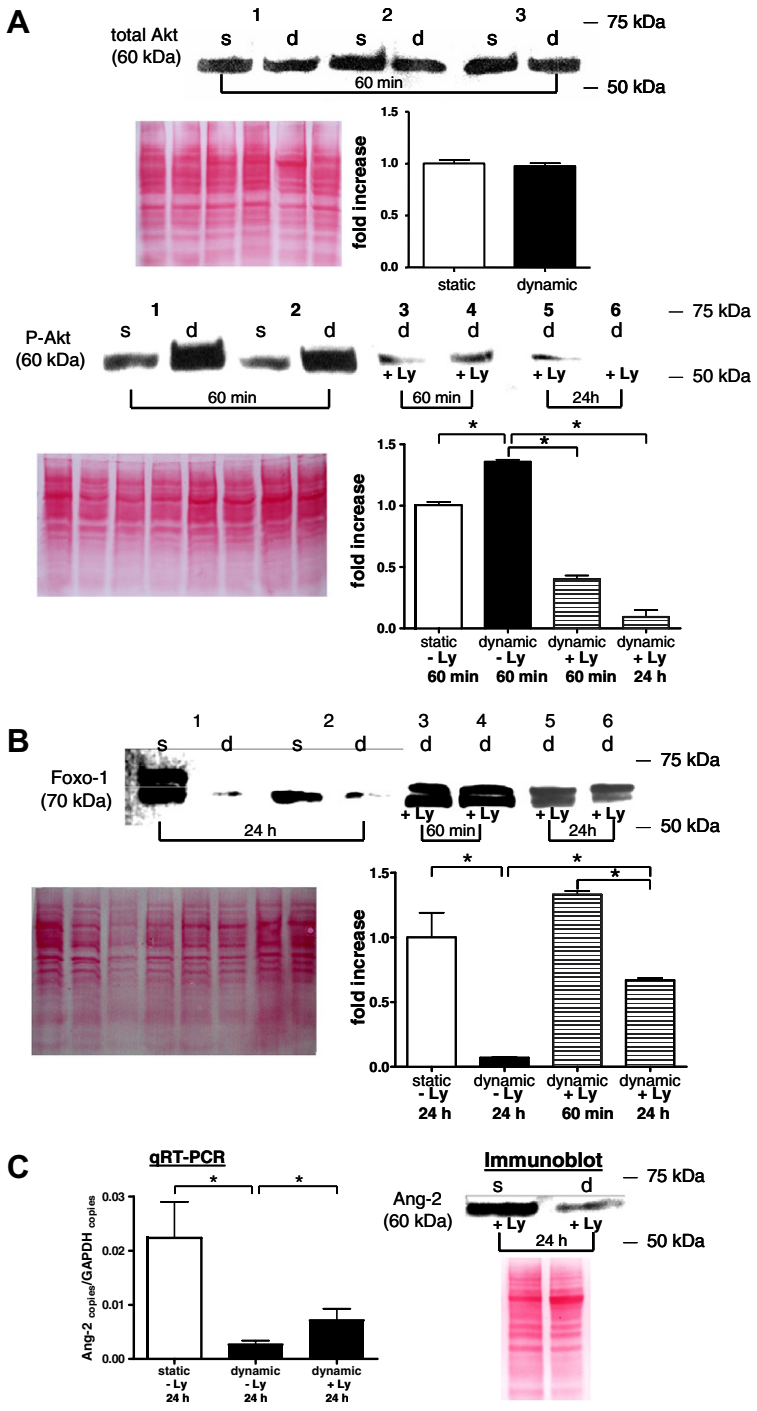

Fig. 4. Influence of PI3K-inhibitor LY-294002 on protein levels of Akt and P-Akt(panel A), Foxo-1 (panel B) as well as on protein and mRNA-level of Ang-2 (panel C) under shear stress. HUVEC were kept under static (s) or dynamic conditions (d: 6 dyn/cm^2, 60 min or 24 h) or first treated with LY-294002 (10 μM; +Ly) and then exposed to shear stress (panels A–C). Paired samples were analyzed by immunoblotting (panels A–C) or quantitative RT-PCR (panel C). Ponceau-stainings are shown as loading controls. Densitometric data are given as means ± S.D. of three independent experiments, $^*P \leqslant 0.05$.

4. Discussion

This study shows mRNA and protein downregulation and phosphorylation (i.e. inactivation) of Foxo-1 by shear stress in endothelial cells. Suppression of Foxo-1 is accompanied by lower transcription of its target gene ANGPT2, coding for Ang-2, a well known receptor antagonist of endothelial Tie2 [5,6]. This suppression of Ang-2 should lead to an activation of the endothelial Tie2 system. Moreover, Tie2 itself was upregulated by shear stress. Shear stress exerted by the flowing blood and acting on endothelial cells could thus regulate blood vessel growth and maturation via the Ang-2/Tie2-system and Foxo-1 as a transcription factor for Ang-2.

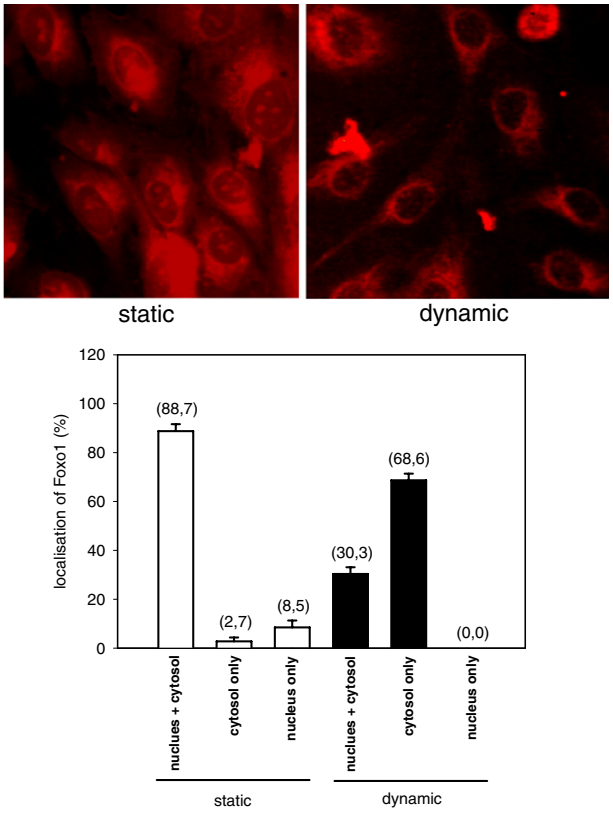

Fig. 5. Translocation of Foxo-1 in HUVEC by shear stress. HUVEC were kept under static control conditions or exposed to shear stress (6 dyn/cm^2, 60 min) and analyzed for the localisation of Foxo-1 by immuno fluorescence microscopy. Three independent experiments were performed and a total of 161 cells analyzed under static and 175 under dynamic conditions. Mean values (in brackets) ± S.D., $n = 3$.

We applied a shear force of up to 6 dyn/cm^2, a value between the typical average level in human venous (≈1 dyn/cm^2) and arterial vessels (≈10 dyn/cm^2) [10,11]. This shear force, which can be present at every part of the vascular tree, had been chosen to more generally discriminate well perfused (mature) and non-perfused (sprouting or occluded) vessels.

Following 24 h of shear stress, the expression of Foxo-1-mRNA was significantly reduced. The inhibition of Foxo-1 by shear stress thus occurs in part on the mRNA level either via transcriptional regulation of the gene or post-transcriptional regulation of mRNA stability. Although the difference in the expression of Foxo-1 between dynamic and static conditions became smaller with proceeding time, it remained statistically significant for at least 24 h. These results were confirmed by measuring a decrease of the total Foxo-1 protein amount following 24 h of shear stress. Thus, the observed effect is relatively long lasting, and shear stress-dependent suppression of Foxo-1 would be well suited to affect structural reorganisation of vascular networks in response to changing hemodynamic forces.

Additional inactivation of Foxo-1 by shear stress (6 dyn/cm^2, 24 h) was achieved through its phosphorylation. The biological significance of these findings was supported by a decreasing level of p27 Kip1 protein, which is a known target gene of Foxo-1 [4]. This is in agreement with Li et al. [12], who found an increase in the phosphorylation of Foxo-1 in bovine aortic endothelial cells following 2 h of shear stress exposure (8 dyn/cm^2 average) in stiff tubes with pulsatile flow. This phosphorylation was further increased if compliant tubes were used suggesting an additional effect of stretch. Stretch-induced phosphorylation of Foxo-1 has indeed been reported in many other cell systems [13,14]. Since phosphorylation of Foxo-1 leads to nuclear exclusion, the translocation of Foxo-1, shown in Fig. 5, would support the biological relevance of its phosphorylation in the presented model system. This is in agreement with similar translocations of Foxo-1 shown as a consequence of Akt-activation by copper or zinc ions [15] or inactivation of Akt by wortmannin [16].

Activation of Akt was convincingly achieved through shear stress, as it had been shown by others before [17]. Phosphorylation of Foxo-1 proved to be almost completely Akt-dependent. Experiments using PI3K inhibitor LY-294 0002 were performed to answer the question to which part transcriptional suppression of Foxo-1 and its posttranscriptional inactivation contribute to Foxo-1-dependent regulation of Ang-2. Since Foxo-1 was downregulated by prolonged shear stress, even during inhibition of PI3K and Akt, this should therefore be due to transcriptional regulation. In addition, only about

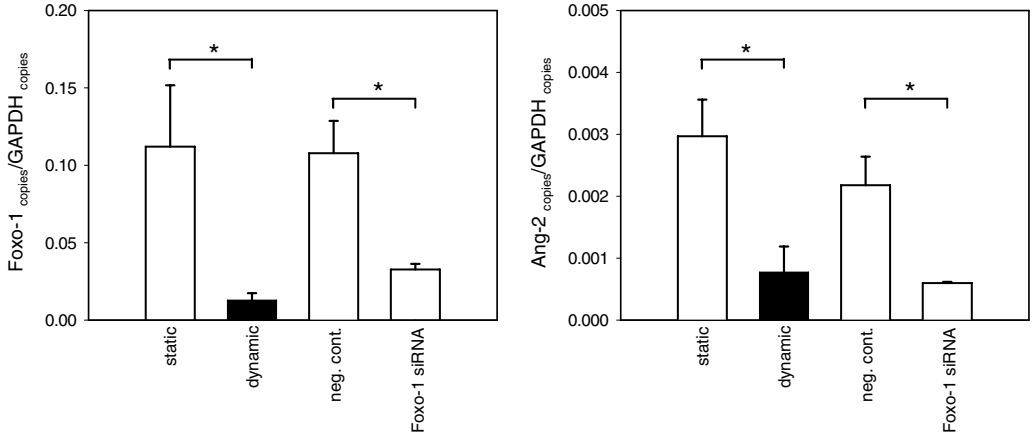

Fig. 6. Expression of Foxo-1- (left panel) and Ang-2-mRNA (right panel) in EAhy926-cells as analyzed by real time RT-PCR. Cells were cultured under static or dynamic (6 dyn/cm^2, 24 h) conditions or under static conditions transfected with siRNA against Foxo-1 or a negative control siRNA. Mean ± S.D., $n = 3$, $^*P \leqslant 0.05$.

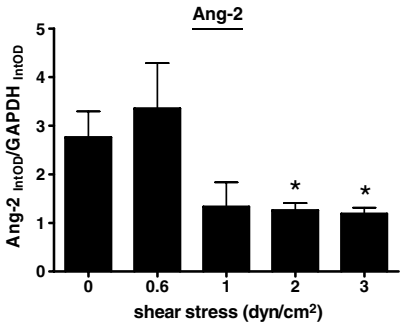

Fig. 7. Suppression of Ang-2 as a function of shear stress. HUVEC were exposed for 24 h to varying shear forces as indicated and analysed by RT-PCR. Data are given as means ± S.D. of three independent experiments, $^{*}P \leqslant 0.05$.

one-third of Ang-2 downregulation by 24 h of shear stress were inhibited through LY-294002. So we conclude, that posttranscriptional inactivation of Foxo-1 through its phosphorylation by shear stress-activated Akt is predominant during early time points after the onset of flow, but becomes less important for longer time periods when the regulation is taken over by transcriptional mechanisms.

Suppression of Ang-2 by shear stress is in agreement with an earlier differential display analysis [7]. Since siRNA against Foxo-1 not only downregulated Foxo-1-mRNA but Ang-2-mRNA as well, suppression of Ang-2 can obviously be achieved by a suppression of Foxo-1. This is in agreement with former studies which already showed expression-regulation of Ang-2 through Foxo-1 [5,16]. The relatively low amount of shear stress at which suppression of Ang-2 occurred, is not well suited to discriminate between different parts of the vascular system but between flow and no-flow conditions. Whereas suppression of Ang-2 by stretch is a transient phenomenon [18], the shear stress effect is more sustained. On the opposite, this predicts upregulation of Ang-2 in sprouting capillaries which has been shown following middle cerebral artery occlusion [19], and would then facilitate vascular endothelial growth factor (VEGF)-induced angiogenesis [6]. During sprouting, the no-flow situation will be additionally supported by hypoxia and VEGF, both of which are known to increase the concentration of Ang-2 [20]. So, in sprouting type of angiogenesis, VEGF and Ang-2 (i.e. inhibition of Tie-2) would cooperate through coupled regulation mechanisms, no-flow and hypoxia.

Ang-1 induced activation and phosphorylation of endothelial Tie2 can be prevented by Ang-2 [6]. Thus, the increased phosphorylation of Tie2 under shear stress as seen by us and others [21] may be achieved through downregulation of Ang-2 mainly. However, since the expression of Tie2 increases during the exposure of HUVEC to shear stress, also shown by others on the mRNA level [22], we cannot conclude from our experiment which part of the increase in phosphorylated Tie2 is due to an increase in its phosphorylation rate. Alternatively, Ang-2 itself has clearly been shown to increase Tie2 phosphorylation in a Foxo-1-dependent manner [16]. In this case, one should expect Tie2 phosphorylation to be downregulated while Ang-2 is suppressed under shear stress. However, the data presented here show the opposite. This might be due to the fact, that Daly et al. investigated a system, in which the expression of Ang-2 had been experimentally increased above basal levels in HUVEC cultured without flow exposure, whereas it was decreased below basal levels in our model system. The resulting activation of the endothelial Tie2 system would promote vessel maturation and maintenance under flow conditions. By contrast, the opposite regulation could be involved to prune blood vessels in the absence of both, blood flow and hypoxia.

By now, we do not know how the upregulation of Tie2 is achieved by increased shear stress. Own experiments with specific inhibition of phospholipase C completely abolished the shear stress-induced expression of Tie2, but had no effect on the downregulation of Ang-2 (not published).

Using the prazosin model in mice, increased capillary wall shear stress has been shown to increase endothelial VEGF concentration [23]. However, sprouting does not occur in this model but splitting [2]. So the splitting type of angiogenesis probably occurs when the VEGF- and the Tie2-system are both activated above their steady state levels.

In summary, our study shows that Foxo-1, Ang-2, and Tie2 are regulated by shear stress acting on endothelial cells. This will have implications for the regulation of different types of angiogenesis and for the development of pharmacological targets to therapeutically modulate angiogenesis in states of altered blood flow.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.01.028.

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