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# Shear stress-induced activation of the AMP-activated protein kinase regulates FoxO1a and angiopoietin-2 in endothelial cells

**Madhulika Dixit[1†], Elke Bess[1], Beate Fisslthaler[1], Frauke V. Härtel[2], Thomas Noll[2], Rudi Busse[1], and Ingrid Fleming[1*]**

[1]*Vascular Signalling Group, Institut für Kardiovaskuläre Physiologie, Johann Wolfgang Goethe Universität, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany and* [2]*Physiologisches Institut, Justus Liebig Universität, D-35392 Giessen, Germany*

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**Aims** Phosphorylation of forkhead box O (FoxO) transcription factors induces their nuclear exclusion and proteosomal degradation. Here, we investigated the effect of fluid shear stress on FoxO1a in primary cultures of human endothelial cells and the kinases that regulate its phosphorylation.
**Methods and results** Shear stress (12 dynes/cm$^2$) elicited the phosphorylation, nuclear exclusion, and degradation of FoxO1a. Inhibition of Akt signalling using either a dominant negative (DN) mutant of Akt or downregulation of Gab1 largely failed to affect the shear stress-induced changes in FoxO1a, while a DN-AMP-activated protein kinase (AMPK) abrogated its shear stress-induced phosphorylation and degradation. Similar effects were observed using the AMPK inhibitor compound C. Moreover, in an *in vitro* assay, the AMPK directly phosphorylated FoxO1a. As FoxO1a regulates the expression of angiopoietin-2 (Ang-2), we determined the role of shear stress and the AMPK in this phenomenon. Not only did the DN-AMPK increase the expression of Ang-2 in cells maintained under static conditions, it also abrogated the shear stress-induced decrease in FoxO1a and Ang-2 protein levels. Functionally, Ang-2 sensitizes endothelial cells to the effects of tumour necrosis factor (TNF)-$\alpha$, and DN-AMPK increased basal endothelial cell E-selectin expression and permeability as well as the increase induced by TNF-$\alpha$.
**Conclusion** These data indicate that the AMPK activated by fluid shear stress is a novel regulator of FoxO1a phosphorylation and protein levels. Moreover, as the AMPK-dependent phosphorylation and degradation of FoxO1a attenuates Ang-2 expression and protects against the pro-inflammatory actions of TNF-$\alpha$, this kinase may be a useful target to prevent the progression of vascular diseases.

## 1. Introduction

Endothelial cells are constantly exposed to mechanical forces such as pulsatile stretch and fluid shear stress. A number of signalling cascades are activated following the application of fluid shear stress to endothelial cells including the phosphorylation and activation of protein kinases such as the Src family of tyrosine kinases[1] and the serine kinase Akt.[2] Although no single specific mechanosensor has been identified to-date, fluid shear stress initiates the rapid phosphorylation of molecules such as the vascular endothelial cell growth factor (VEGF) receptor 2,[3] as well as of the platelet-endothelial cell adhesion molecule (PECAM)-1[4,5] which then modulate the activity of downstream signalling molecules such as Akt. A great deal is now known about the consequences of Akt activation, in particular its role in the prevention of apoptosis,[2] the regulation of forkhead (FoxO) transcription factors,[6,7] and in the phosphorylation of the endothelial nitric oxide synthase (eNOS).[8] However, the cellular consequences of other shear stress-activated kinases are less clear. One example of such a kinase is the AMP-activated protein kinase (AMPK), which can be activated by fluid shear stress via a pathway that is independent of PECAM-1 and distinct from that resulting in the activation of Akt.[5]

There are close links between the activation of Akt and that of the AMPK, inasmuch as AMPK activation is required for the maintenance of pro-angiogenic Akt signalling in endothelial cells exposed to hypoxia[9] and to adiponectin.[10]

* Corresponding author. Tel: +49 69 6301 6972; fax: +49 69 6301 7668.
*E-mail address*: fleming@em.uni-frankfurt.de
† Present address. Department of Biotechnology, Indian Institute of Technology Madras, Chennai 600036, India.

On the other hand, Akt is reported to be a negative regulator of the AMPK in a mouse embryonic fibroblast cell line[11] as well as in the heart.[12] There is also a potential overlap in the actions of the two kinases as both are reported to phosphorylate eNOS[13] and FoxOs can be phosphorylated by Akt[6] as well as by AMPK-activating stimuli such as glucose starvation and AICAR.[14] As its name suggests, the AMPK is activated by increased intracellular concentrations of AMP, and is generally described as a 'metabolite-sensing kinase' that can be stimulated by heat shock, vigorous exercise, hypoxia/ischaemia, and glucose starvation.[15] However, it is now apparent that the AMPK can be activated in endothelial cells in the absence of marked changes in AMP by additional intracellular signals including upstream kinases such as LKB1 and $Ca^{2+}$/calmodulin-dependent kinase kinase[16] as well as by reactive oxygen and nitrogen species.[17–19]

The aim of the present investigation was to determine the functional significance of the shear stress-induced activation of Akt and AMPK concentrating on the role of these two kinases on the phosphorylation of FoxO1a and the subsequent changes in the expression of the FoxO1a-regulated gene, angiopoietin-2 (Ang-2).[20,21]

## 2. Methods

### 2.1. Materials

Antibodies for western blotting and immunochemistry directed against phospho-Ser473Akt, Akt, phospho-Thr172AMPK, phospho-Thr32FoxO1a (FKHR), FoxO1a (FKHR), and Gab1 were purchased from Cell Signaling (New England Biolabs, Frankfurt, Germany) and those recognizing Ang-2 and c-myc from Santa Cruz Biotechnology (Heidelberg, Germany). All other substances were from Sigma (Deisenhofen, Germany).

### 2.2. Cell culture

Human umbilical vein endothelial cells were isolated and cultured as described previously.[22] The use of human material in this study conforms to the principles outlined in the Declaration of Helsinki. Confluent cultures of endothelial cells were washed twice in culture medium containing 2% foetal calf serum (FCS) and were either maintained under static conditions or exposed to shear stress (12 dynes/cm$^2$) in a temperature-controlled cone-plate viscosimeter, as described.[23] In some experiments, permeability across the endothelial monolayer was determined in a two-compartment system separated by a filter as described.[24]

### 2.3. Plasmids and transfection

Endothelial cells (80–90% confluent) were transfected with pcDNA3.1 or a dominant negative (DN)-Akt mutant (K179M; provided by Stefanie Dimmeler, Frankfurt, Germany) using Transpass D2 (New England Biolabs, Frankfurt, Germany) as reported.[25]

### 2.4. Adenoviral transduction of endothelial cells

Subconfluent endothelial cells were infected with adenoviruses (kindly provided by Ken Walsh, Boston, USA) to overexpress constitutively active (CA)-AMPK,[26] or DN-AMPK[27] as described.[28] Briefly, endothelial cells (70–90% confluent) were incubated in MCDB131 containing 0.1% BSA for 6 h. Prior to infection (30 min), adenoviruses (m.o.i of 2) were incubated with a peptide derived from the homoeobox gene antennapedia (100 μmol/L, Eurogentec, Seraing, Belgium) in 50 μL. The mixture was then applied to endothelial cells in MCDB131 medium containing 0.1% BSA for 4 h. Thereafter, the cells were washed four times with MCDB131 and cultured for an additional 48 h in the presence of 2% FCS.

### 2.5. Small interfering RNA

A commercially available kit (SMART pool; Upstate, Hamburg, Germany) was used according to the manufacturer's instructions. Confluent primary cultures of endothelial cells were starved of serum for 6 h prior to transfection with Gab1 small interfering RNA (siRNA) or FoxO1a siRNA and the transfection reagent SilMPORTER (Upstate) as described.[25,29]

### 2.6. *In vitro* phosphorylation of FoxO1a

AMPK was immunoprecipitated from endothelial cells exposed to solvent (culture medium) or pentobarbital (2 mmol/L, 10 min) to activate AMPK. FoxO1a was also immunoprecipitated from unstimulated endothelial cells and used as a substrate for an AMPK kinase assay as described.[19] The kinase reaction was separated by SDS–PAGE and radioactivity was detected by exposure of the gel to an X-ray film.

### 2.7. Immunoblotting

Cells were either harvested in SDS–PAGE sample buffer or lysed in buffer containing Tris/HCl (pH 7.5; 50 mmol/L), NaCl (150 mmol/L), NaF (100 mmol/L), $Na_4P_2O_7$ (15 mmol/L), $Na_3VO_4$ (2 mmol/L), leupeptin (2 μg/mL), pepstatin A (2 μg/mL), trypsin inhibitor (10 μg/mL), phenylmethylsulfonyl fluoride (PMSF; 44 μg/mL), and Triton X-100 (1% v/v), left on ice for 10 min and centrifuged at 10 000 *g* for 10 min. Cell supernatants were heated with SDS–PAGE sample buffer and separated by SDS–PAGE as described.[29] Proteins were detected using their respective antibodies and enhanced chemiluminescence using a commercially available kit (Amersham, Germany). To assess the phosphorylation of proteins, either equal amounts of protein from each sample were loaded twice and one membrane incubated with the phospho-specific antibody and the other with an antibody recognizing total protein, or blots were re-probed with the appropriate antibody.

### 2.8. Immunostaining

Endothelial cells were grown on glass slides and exposed to fluid shear stress. Thereafter, the cells were fixed with paraformaldehyde (4% in PBS), incubated in PBS containing glycine (2%) for 10 min, and permeabilized with Triton X-100 (0.2%). After blocking, cells were incubated with an anti-FoxO1a antibody (1:150 dilution) followed by an Alexa dye-coupled secondary antibody (Molecular Probes, Gottingen, Germany). Preparations were mounted and viewed using a confocal microscope.

### 2.9. Statistics

Values are expressed as the mean ± SEM and statistical evaluation was performed using Student's *t*-test for unpaired data and one-way ANOVA or ANOVA for repeated measures followed by a Bonferroni *t*-test where appropriate. Values of $P < 0.05$ were considered statistically significant.

## 3. Results

### 3.1. Effect of shear stress on phosphorylation of Akt, AMPK, and FoxO1a

In confluent primary cultures of human endothelial cells, fluid shear stress (12 dynes/cm$^2$) elicited the phosphorylation of Akt on Ser473 and AMPK on Thr172, which was maintained as long as the stimulus was applied, i.e. up to 72 h (*Figure 1A*). In line with reports that FoxO1 is an Akt substrate, Akt phosphorylation was paralleled by the phosphorylation of FoxO1a and by a decrease in FoxO1a protein levels (*Figure 1A*). Indeed, FoxO1a was clearly present in

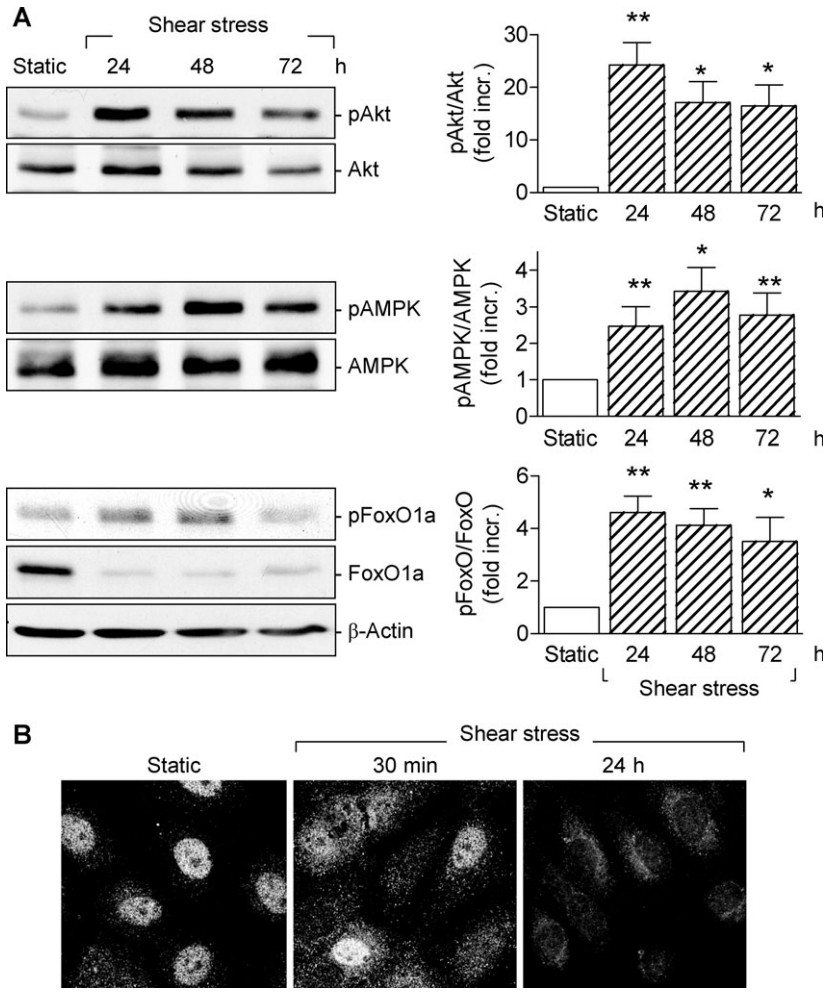


**Figure 1** Effect of shear stress on phosphorylation of Akt, AMPK, and FoxO1a and the exclusion of FoxO1a from the nucleus. Endothelial cells were either maintained under static conditions or exposed to shear stress of 12 dynes/cm$^2$ for up to 72 h. (*A*) The effect of shear stress on the phosphorylation of Akt, AMPK, and FoxOa1 and on FoxOa1 protein levels as detected by western blotting with phospho-specific antibodies. Equal loading was confirmed by blotting the membranes for β-actin. The bar graphs summarize data obtained in six independent experiments; $^*P < 0.05$ and $^{**}P < 0.01$ vs. static. (*B*) Effect of shear stress on the intracellular localization of FoxO1a. Cells were exposed to shear stress for up to 24 h, fixed and stained with an antibody against FoxO1a. Identical results were obtained in three independent experiments.

the nucleus of endothelial cells maintained under static conditions, and shear stress elicited a rapid (i.e. within 30 min) nuclear exclusion of the transcription factor (*Figure 1B*).

### 3.2. Contribution of Akt and AMPK to the shear stress-induced phosphorylation of FoxO1a

To determine the involvement of Akt in the phosphorylation of FoxO1a in response to fluid shear stress, we assessed the effects of a DN-Akt mutant and the consequences of downregulating the expression of the scaffolding protein Gab1, which prevents the shear stress-induced activation of Akt.[29,30]

The DN-Akt mutant (overexpression verified by its Myc-tag) did not influence the shear stress-induced phosphorylation and degradation of FoxO1a (*Figure 2A*). These data were confirmed in experiments, in which Akt activation was suppressed by downregulating the expression of the scaffolding protein Gab1. Although Gab1 siRNA markedly suppressed the shear stress-induced phosphorylation and thus activation of Akt (*Figure 2B*), this intervention failed to significantly attenuate the phosphorylation and degradation of FoxO1a (*Figure 2B*) or the shear stress-induced phosphorylation of AMPK (*Figure 3A*). In line with these data indicating that the AMPK may also mediate the phosphorylation of FoxO1a, the AMPK inhibitor, compound C attenuated the shear stress-induced nuclear exclusion of FoxO1 (data not shown) as well as the shear stress-induced degradation of FoxO1a protein (*Figure 3B*).

To more convincingly demonstrate the involvement of AMPK in the regulation of FoxO1a phosphorylation, we determined the ability of the AMPK immunoprecipitated from basal or stimulated endothelial cells to phosphorylate FoxO1a *in vitro*. Activation of the AMPK resulted in a $5.7 \pm 1.7$-fold ($P < 0.01$, $n = 4$) increase in the phosphorylation of FoxO1a (*Figure 3C*). Next, endothelial cells were infected with either a control virus or a DN-AMPK mutant. Although endothelial cells treated with the control virus responded to shear stress with an increased phosphorylation of FoxO1a, this did not occur in cells overexpressing the DN-kinase (*Figure 3D*). Moreover, the DN-AMPK increased the expression of FoxO1a in endothelial cells maintained under static conditions. The opposite effect was observed in cells overexpressing a CA-AMPK and FoxO1a levels were

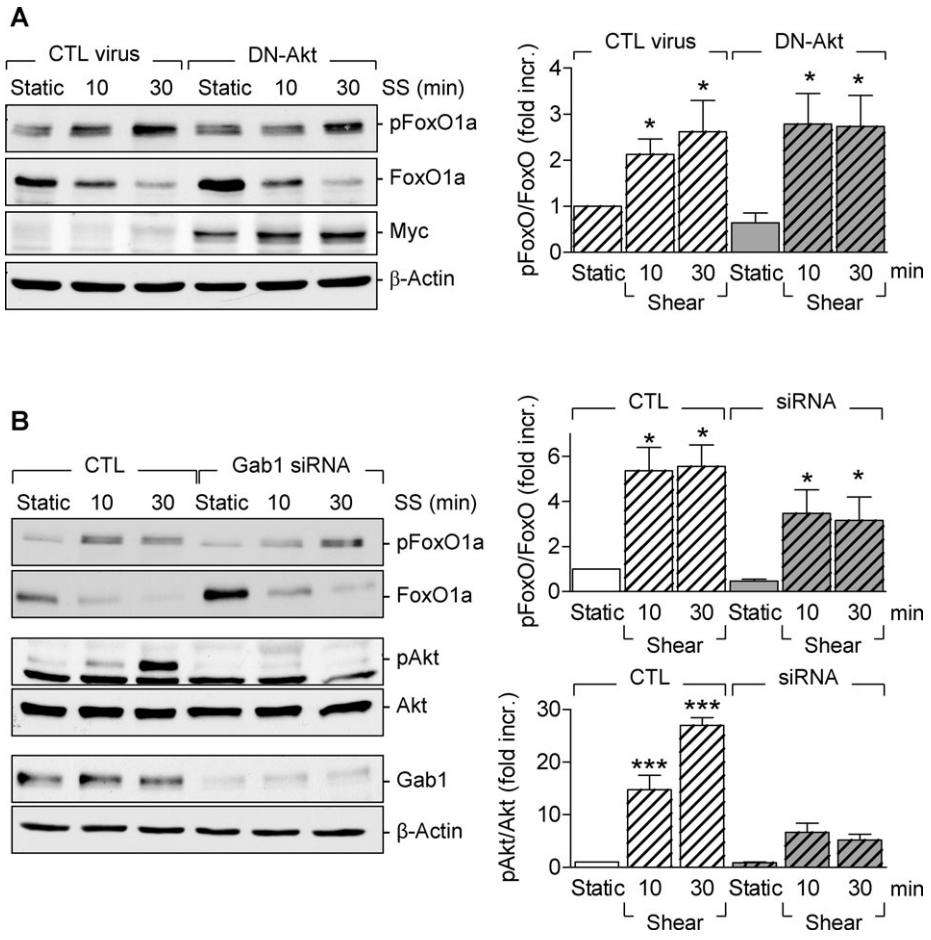


**Figure 2** Effect of preventing Akt activation on the shear stress-induced phosphorylation of FoxO1a. (*A*) Endothelial cells were transfected with a control (CTL) plasmid or a plasmid encoding the dominant negative (DN)-Akt mutant 48 h prior to stimulation with shear stress for up to 30 min. The phosphorylation and expression of FoxO1a was determined using specific antibodies and overexpression of the DN-Akt was verified by probing for its Myc-tag. Equal loading of the lanes was confirmed by probing for β-actin. (*B*) Endothelial cells were treated with CTL oligonucleotides or Gab1 siRNA for 72 h prior to stimulation with shear stress. The western blots show the effect of the Gab1 siRNA on Gab1 protein levels as well as on the phosphorylation and expression of Akt and FoxO1a. The bar graphs summarize the data obtained in three to four independent experiments; $^{*}P < 0.05$, and $^{***}P < 0.001$ vs. static conditions.

slightly decreased (by $32 \pm 5\%$, $n = 4$) below the levels detected in endothelial cells treated with a control virus (*Figure 4A*).

### 3.3. Effect of AMPK activation and fluid shear stress on the expression of Ang-2

FoxO1a regulates the expression of a number of genes in endothelial cells including that of Ang-2.[20,21] Therefore, we assessed the consequences of the DN- and CA-AMPK mutants on its expression. The effects of the AMPK mutants on Ang-2 expression mirrored those on FoxO1a protein levels such that the DN-AMPK significantly increased Ang-2 protein, whereas the CA-AMPK mutant markedly decreased Ang-2 protein expression (*Figure 4B*).

Although Ang-2 expression was clearly evident in endothelial cells maintained under static conditions, the application of fluid shear stress-elicited a downregulation of the protein (*Figure 5A*). Over the same period (72 h), there was a significant upregulation in the expression of eNOS; the eNOS/PECAM ratio was increased by $12.4 \pm 2.6$-fold over levels detected in cells maintained under static conditions ($n = 15$, $P < 0.001$). The shear stress-induced downregulation of Ang-2 expression was prevented by the DN-AMPK mutant (*Figure 5B*). Moreover, while a control siRNA failed to influence the basal expression of Ang-2 or the decrease elicited by shear stress, FoxO1a siRNA abrogated basal Ang-2 expression (*Figure 5C*). Under these conditions, shear stress was without additional effect.

As Ang-2 is an autocrine regulator of endothelial cell inflammatory responses and sensitizes endothelial cells to the pro-inflammatory actions of TNF-α,[31] we assessed the consequences of manipulating AMPK activity on the basal and TNF-α-induced expression of E-selectin as well as on changes in cell permeability. Under basal conditions, the overexpression of the DN-AMPK significantly increased endothelial cell E-selectin expression (*Figure 6A*). Overexpression of the CA-AMPK, on the other hand, did significantly alter E-selectin expression. Endothelial cell stimulation with TNF-α (10 ng/mL) significantly increased endothelial cell E-selectin levels in cells treated with a control virus and this effect was potentiated in cells overexpressing the DN-AMPK and attenuated (although not significantly) in cells overexpressing the CA-AMPK (*Figure 6A*).

Overexpression of the DN-AMPK mutant was associated with an increased basal permeability of endothelial cell monolayers ($P < 0.001$, $n = 4$), compared with cells treated with the control virus. Moreover, the TNF-α (10 ng/mL)-induced

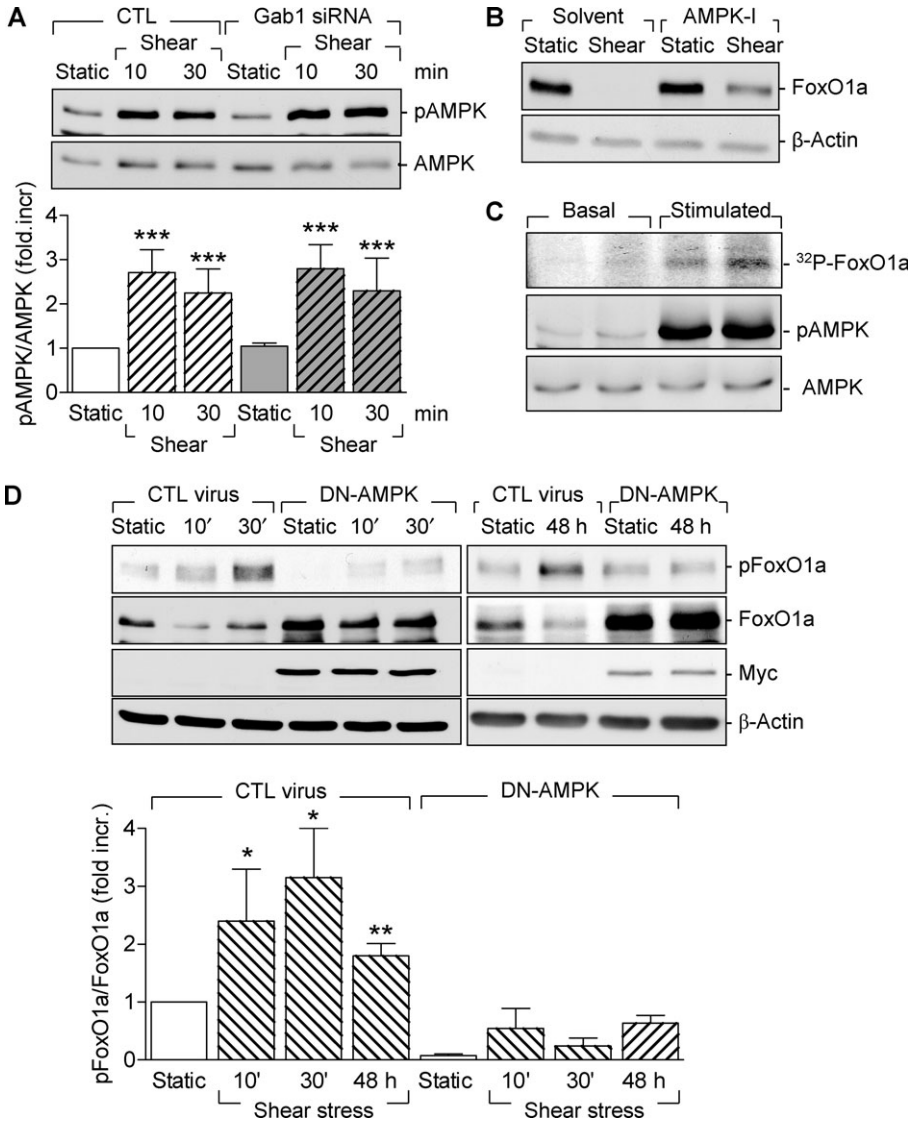


**Figure 3** Effect of shear stress on the phosphorylation of AMPK and the effect of AMPK inhibition on the phosphorylation and degradation of FoxO1a. (*A*) Effect of control oligonucleotides (CTL) or Gab1 siRNA on the shear stress-induced phosphorylation of AMPK. (*B*) Effect of the AMPK inhibitor (AMPK-I) compound C (10 μmol/L) on the shear stress (30 min)-induced degradation of FoxO1a. Equal loading of the lanes was confirmed by probing for β-actin. (*C*) AMPK kinase assay with FoxO1a as substrate. The AMPK was immunoprecipitated from endothelial cells exposed to solvent or pentobarbital (2 mmol/L, 10 min) to activate AMPK. The phosphorylation of FoxO1a ($^{32}$P) is shown in the upper panel; the immunoblots demonstrate the activation of the AMPK by pentobarbital and the use of equal amounts of AMPK in the kinase reaction. (*D*) Effect of the dominant negative (DN)-AMPK mutant on the shear stress-induced phosphorylation and expression of FoxO1a. Endothelial cells were infected with either a CTL virus or an adenovirus encoding the DN-AMPK mutant 48 h prior to exposure to shear stress for up to 48 h. The phosphorylation of FoxO1a was determined via western blotting with a phospho-specific antibody. Overexpression of the DN-AMPK was verified by its Myc-tag. Blots were re-probed for total FoxO1a protein and equal loading of each lane was confirmed by probing for β-actin. The bar graphs summarize the data obtained in three to nine independent experiments; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. static.

increase in endothelial cell permeability was more rapid (significant differences in permeability were detected 34 min after the addition of TNF-α to DN-AMPK expressing cells and after 42 min in cells treated with the control virus) and more pronounced in DN-AMPK-expressing cells (*Figure 6*).

## 4. Discussion

The results of the present investigation have identified FoxO1a as a downstream target of the AMPK activated by the application of fluid shear stress to human endothelial cells. The AMPK-dependent phosphorylation and degradation of FoxO1a were associated with a decrease in the expression of the FoxO1a-regulated gene Ang-2. Moreover, a DN-AMPK mutant prevented the shear stress-induced downregulation of Ang-2 and increased endothelial cell permeability.

Members of the FoxO family of transcription factors regulate the expression of numerous genes involved in the cell cycle, apoptosis, differentiation, development, DNA repair, and the cellular response to oxidative stress.[32,33] FoxO factors are generally classed as downstream targets of the protein kinase Akt, which phosphorylates and renders them transcriptionally inactive by promoting their nuclear exclusion and subsequent proteosomal degradation.[34] We have recently reported a potential link between the AMPK and FoxO1a and its subsequent effect on the expression

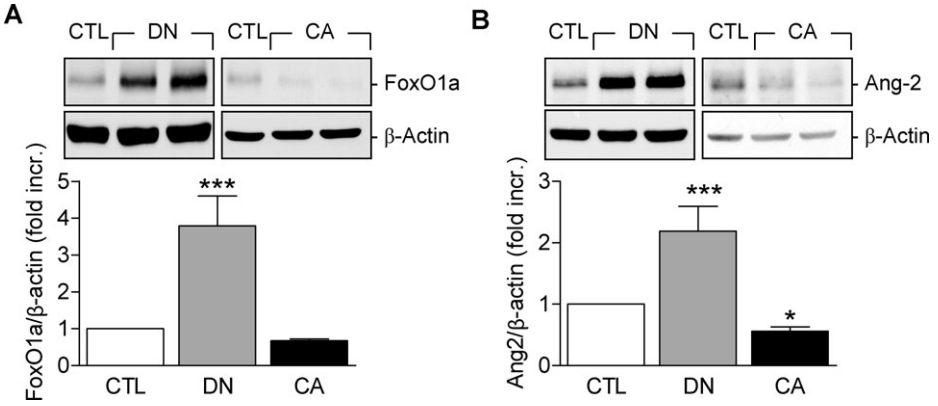


**Figure 4** Effect of dominant negative (DN)- and constitutively active (CA)-AMPK on the expression of FoxO1a and Ang-2 protein. Endothelial cells were infected with either a control (CTL) virus or with adenovirus encoding either the DN or CA mutants of AMPK for 48 h. (*A*) FoxO1a and (*B*) Ang-2 expression was assessed by western blot analyses and normalized to that of β-actin. The bar graphs summarize the data obtained in four to five independent experiments; $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. CTL.

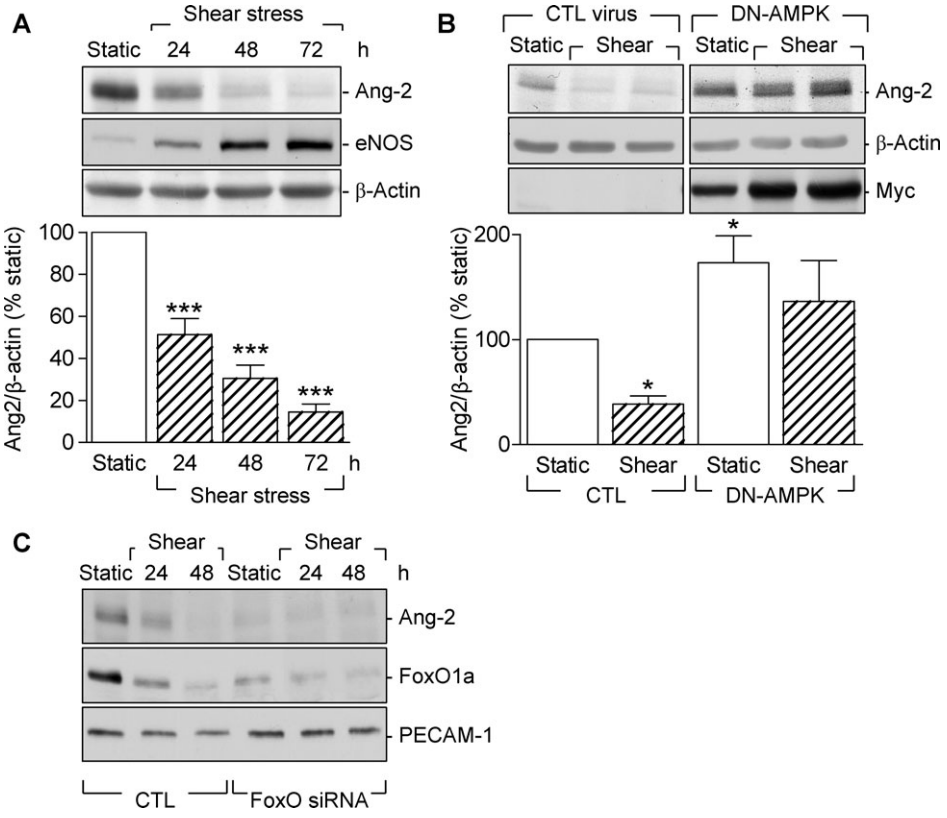


**Figure 5** Effect of shear stress and dominant negative (DN)-AMPK on the expression of Ang-2. (*A*) Endothelial cells were exposed to shear stress for up to 72 h and Ang-2 as well as eNOS protein expression was assessed by western blot analysis. (*B*) Effect of overexpression of DN-AMPK on shear stress-induced downregulation of Ang-2 expression. Endothelial cells were treated with control (CTL) virus or DN-AMPK for 48 h prior to stimulation with shear stress for 48 h. Equal loading of individual lanes was verified with β-actin and overexpression of the DN-AMPK was verified by its Myc-tag. The bar graphs summarize the data obtained in four to nine independent experiments; $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. the static control. (*C*) Effect of a CTL siRNA and a FoxO1a-specific siRNA on the expression of Ang-2. Experiments were performed under basal conditions as well as following the application of shear stress for up to 48 h. Identical results were obtained in two additional experiments.

and activity of the hydroxy-methylglutaryl coenzyme A reductase in endothelial cells.[19] However, the molecular interventions performed in the present investigation, including the overexpression of a DN-Akt, downregulation of Gab1 as well as the overexpression of CA and DN mutants of the AMPK clearly demonstrate that the AMPK can also (in addition to Akt) act as an important regulator of FoxO transcription factors in endothelial cells, especially in response to cell stimulation by fluid shear stress.

The AMPK is widely accepted as a key regulator of cellular energy homeostasis, however, the physiological functions of AMPK are likely to be more complex than initially assumed as the regulation of cellular energy levels seems to be only one aspect of AMPK signalling.[35] Certainly the AMPK can also

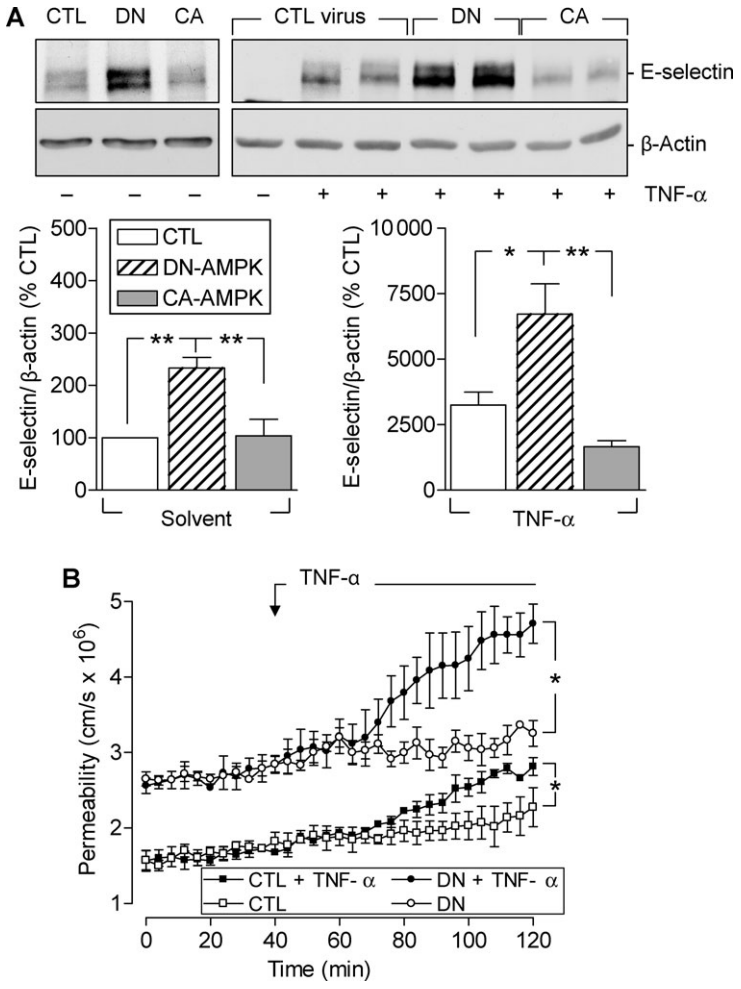


**Figure 6** Effect of dominant negative (DN)-AMPK on the TNF-α-induced increase in endothelial cell E-selectin expression and permeability to albumin. (*A*) Endothelial cells were infected with either a control (CTL) virus or an adenovirus encoding either the DN- or constitutively active (CA)-AMPK mutant and after 48 h the expression of E-selectin was assessed in response to either solvent or TNF-α (10 ng/mL, 6 h). Given the very large differences in basal and TNF-α-stimulated E-selectin expression in endothelial cells, the blots shown in the left and right panels were exposed for different times. E-selectin levels are normalized to that observed in cells infected with the CTL virus and treated with solvent. The bar graph summarizes the data obtained in four to five independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the solvent-treated CTL. (*B*) Endothelial cells were infected with either a CTL virus or an adenovirus encoding the DN-AMPK mutant and after 48 h the permeability of the monolayer to albumin in the absence and presence of TNF-α (10 ng/mL) was determined. The graph summarizes the data obtained in four independent experiments and two different cell batches, each performed in quadruplicate; $^{*}P < 0.05$, vs. the absence of TNF-α.

affect gene expression, in particular the expression of genes involved in metabolism.[35] Although the kinase is known to interact with several transcription factors including; NFκ-B,[36] p300,[37] HNF4α[38] and now FoxO1a, little is known about its interaction with kruppel-like factor 2 (KLF-2) which currently appears to play a dominant role in the endothelial response to mechanical stimulation including the expression of Ang-2.[39] It will certainly be interesting to elucidate whether or not a relationship exists between shear stress, endothelial NO production, the AMPK, and the subsequent activation of KLF-2 and/or inhibition of ATF2.[40] To couple the changes in AMPK and FoxO1a activity with endothelial cell function, we assessed the consequences of shear stress and the CA- and DN-AMPK mutants on Ang-2 expression, the latter protein was chosen as it has recently been identified as a FoxO1a-regulated gene.[20,21] We found that Ang-2 was constitutively expressed in the human endothelial cells studied and that its expression was time-dependently decreased following the application of shear stress. Moreover, the shear stress-induced decrease in Ang-2 expression was largely attenuated in cells expressing the DN-AMPK. Given that the shear stress-induced activation of the AMPK led to a decrease in the phosphorylation and expression of FoxO1a and subsequently to a decreased expression of Ang-2, it is tempting to speculate a role for AMPK in shear stress-stimulated vessel remodelling. Certainly, previous studies have indicated a regulatory function of AMPK in angiogenesis induced by hypoxia/ischaemia,[9] however similar responses in cells exposed to fluid shear stress and the extent of involvement of FoxO1a-regulated genes in these phenomena remain to be investigated.

It is currently unclear how the AMPK is phosphorylated in endothelial cells exposed to shear stress but activation occurs in a PECAM-1 and Akt-independent manner.[5] The nearest relatives of kinases responsible for activation of yeast homologue of AMPK, i.e. SNF-1, are LKB1 and the β-isoform of the calmodulin-dependent protein kinase.[41] However, the activation of AMPK in human umbilical vein endothelial cells is reported to be LKB1-independent and linked to rapid release of nucleotides from activated endothelial cells and the subsequent activation of G-protein-coupled receptors of the P2Y family.[42]

Other alternatives such as mitochondrion-derived reactive oxygen species as well as NO have also been implicated in the activation of the kinase.[18] Indeed NO facilitates the generation of mitochondrial ROS and the subsequent phosphorylation of the AMPK,[18] whereas peroxynitrite, the reaction product of superoxide anions and NO, activates the kinase in endothelial cells.[17]

Ang-2 is reported to contribute to vessel destabilization and remodelling, making the vasculature more plastic and amenable to sprouting (under the influence of VEGF) or regression,[43] as well as to sensitize endothelial cells to the pro-inflammatory actions of TNF-$\alpha$.[31] We therefore also assessed the consequences of manipulating AMPK activity on endothelial cell adhesion molecule (E-selectin) expression as well as permeability. The data obtained indicate that the basal E-selectin levels and permeability as well as the increases elicited by TNF-$\alpha$ were greater in endothelial cells expressing the DN-AMPK, which also expressed significantly more Ang-2 than cells infected with a control virus. During the preparation of this manuscript, results from other groups have provided support for a role of the AMPK in the regulation of cell permeability as the kinase is reported to regulate the formation of epithelial cell tight junctions.[44,45] Unfortunately, the experimental model used did not allow us to determine the effects of fluid shear stress on endothelial cell permeability.

Although shear stress time-dependently decreased the expression of Ang-2, this is certainly not the only mechanism that can contribute to the protection against the inflammatory actions of TNF-$\alpha$ *in vivo*. Indeed, shear stress is known to attenuate inflammatory responses elicited by TNF-$\alpha$ in endothelial cells and to attenuate the expression of the adhesion molecules by a mechanism linked to the shear stress-induced increase in NO production and the inactivation of the transcription factor, NF$\kappa$-B (for review, see ref. 46). To what extent the AMPK is implicated in the shear stress-induced inactivation of NF$\kappa$-B in endothelial cells remains to be determined although there is evidence linking the AMPK to this transcription factor in response to other stimuli.[36,47]

Taken together, our data indicate that the shear stress-induced activation of the AMPK and the subsequent regulation of the transcription factor FoxO1a lead to changes in endothelial cell gene expression, exemplified here by the downregulation of Ang-2. Given the role of Ang-2 in vascular remodelling; this signalling cascade may contribute to remodelling of the vascular network. Moreover, as the AMPK protects against the pro-inflammatory actions of TNF-$\alpha$, this kinase may thus be a useful target to prevent the progression of vascular diseases.

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**Conflict of interest**: none declared.

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