Chapter 3

Shear stress counteracts the pro-inflammatory effects of oxidative stress and TGF-β on endothelial cells by suppressing the TAK1 pathway

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Submitted
Abstract

Aims:
Endothelial dysfunction, characterised by an imbalanced redox homeostasis and pro-inflammatory phenotype, precedes the pathogenesis of cardiovascular disorders. Shear stress increases nitric oxide formation and antagonises endothelial dysfunction by activating anti-inflammatory pathways and suppressing pro-inflammatory pathways. Transforming growth factor-β-activated kinase 1 (TAK1) is a key mediator in both inflammation and (non-canonical) transforming growth factor-β (TGF-β) signalling. The individual roles of TAK1, extracellular-signal-regulated kinase 5 (ERK5) and TGF-β pathways in regulating endothelial phenotype are well characterised, yet an overall understanding of the orchestration of these pathways and their crosstalk with the redox system under shear stress is lacking. We hypothesised that shear stress counteracts the pro-inflammatory effects of oxidative stress and TGF-β1 on endothelial cells by restoring redox balance and repressing the TAK1 pathway through ERK5.

Methods and results:
We here showed in human umbilical vein endothelial cells that TGF-β1 aggravated oxidative stress-mediated pro-inflammatory effects. Our data also demonstrate shear stress activated ERK5 signalling while attenuating canonical TGF-β [activin receptor-like kinase 5 (ALK5)] signalling. Activation of ERK5 by a constitutively active form of MEK5 restored redox balance, but repressed neither ALK5 kinase activity nor the induction of inflammatory mediators. Inhibition of TAK1 strongly suppressed the expression of inflammatory mediators, as did attenuation of mitochondrial reactive oxygen species generation. These results imply shear stress impedes TGF-β1-induced TAK1 signalling through pathways other than ERK5, in a manner independent of redox balance and ALK5 kinase activity.

Conclusions:
In summary, our in vitro studies demonstrate that shear stress restores redox balance via stimulation of the ERK5 pathway, but counteracts the pro-inflammatory effects of TGF-β1 via repression of the TAK1 pathway, independent of ERK5.
Introduction

The endothelium is a monolayer of cells that acts as the regulatory interface between blood and the vessel wall. Given the capability to receive and respond to both biochemical and biomechanical stimuli, it is a key regulator of cardiovascular homeostasis and disorders. Adverse alterations of the endothelial phenotype (endothelial dysfunction) precede the pathogenesis of cardiovascular disorders, particularly atherosclerosis and pulmonary hypertension. The maintenance of a healthy endothelial phenotype relies on a delicate balance between nitric oxide (NO) and reactive oxygen species (ROS). Decreased NO availability, following increased NO degradation by ROS tips the balance of redox system and brings about endothelial dysfunction.

In physiology, the phenotype of endothelial cells is tightly regulated by their responses to mechanical forces, in particular shear stress. Shear stress exerted by laminar blood flow increases NO bioavailability, while reducing ROS production. Therefore, shear stress safeguards endothelial redox homeostasis and counteracts endothelial dysfunction.

The protective effects of shear stress on endothelial cells extend to its inhibition of pro-inflammatory signalling cascades, such as nuclear factor kappa-light-chain-enhancer of activated B cell (NFκB), p38 mitogen-activated protein kinase (MAPK) and c-Jun NH₂-terminal kinase (JNK) pathways. As expected, the expression of inflammatory molecules, such as adhesion molecules and chemoattractants, that are activated by these signalling pathways, are also inhibited by shear stress. Moreover, shear stress also elicits its protective effects through activation of extracellular-signal-regulated kinase 5 (ERK5) and transforming growth factor-β (TGF-β) signalling in embryonic and human umbilical vein endothelial cells. ERK5 signalling downregulates inflammation through induction of the anti-inflammatory transcription factors, Kruppel-like factor 2 (KLF2) or KLF4. TGF-β signalling mediates shear-induced KLF2 expression through the activin receptor-like kinase 5 (ALK5) pathway. While the individual roles of NFkB, p38 MAPK, JNK, ERK5 and TGF-β pathways in endothelial dysfunction are well delineated, an overall understanding of the orchestration of these pathways and their crosstalk with the redox system in the context of relevant haemodynamic forces remain obscure.

The elevated level of TGF-β during vascular injury alters endothelial phenotype. Upon stimulation with TGF-β, the type II receptors (TβRII) couple with the type I receptors (TβRI), i.e. ALK5 and leads to phosphorylation of receptor-regulated small mothers against decapentaplegic (R-SMADs), i.e. SMAD2 and SMAD3. In addition to activating the canonical SMAD pathway, TGF-β also activates the non-canonical TGF-β-activated kinase 1 (TAK1) pathway. TGF-β activates TAK1 in a kinase-independent manner...
involving TβRI, TNF receptor-associated factor 6 (TRAF6) ubiquitin ligase and ubiquitylation.\textsuperscript{18,19} Interaction with TβRI activates E3-ubiquitin ligase activity of TRAF6 and leads to autoubiquitylation of TRAF6. TAK1-binding protein 2 (TAB2) or TAB3 which are adapted to TAK1 binds polyubiquitin chains on TRAF6 and results in polyubiquitylation of TAK1 that accounts for TAK1 activation.\textsuperscript{18,19} TAK1 is a mitogen-activated protein kinase kinase (MAP3K), also known as MAP3K7, which activates p38 MAPK and JNK signalling through mitogen-activated protein kinase kinase 3 (M KK3)/MKK6 and MKK4/7, respectively.\textsuperscript{20} Furthermore, TAK1 also activates NFκB signalling by activating the IκB kinase (IKK) complex.\textsuperscript{21} Activation of TAK1 by inflammatory cytokines induces the expression of inflammatory molecules in endothelial cells.\textsuperscript{9} Surprisingly, the consequences of TAK1 activation for endothelial cells under stimulation of TGF-β and its regulation by shear stress remain elusive.

It appears that shear stress activates ERK5\textsuperscript{7} and ALK5\textsuperscript{11,12} signalling, which is associated with the upregulation of anti-inflammatory transcription factors, whereas oxidative stress\textsuperscript{1,2} and TAK1\textsuperscript{9} signalling promote a pro-inflammatory endothelial phenotype. Intriguingly, the molecular mechanisms by which shear stress regulates the phenotype of endothelial cells upon oxidative stress and stimulation of TGF-β1 are poorly understood. Our earlier studies revealed that shear stress mediates the process of endothelial-to-mesenchymal transition (EndMT) through the ERK5 pathway.\textsuperscript{22} Here, we hypothesised that shear stress counteracts the pro-inflammatory effects of oxidative stress and TGF-β1 on endothelial cells by restoring the redox balance and repressing the TAK1 pathway through ERK5. To test this hypothesis, we performed an \textit{in vitro} study in human umbilical vein endothelial cells (HUVEC) to examine how shear stress, ERK5, ALK5 and TAK1 signalling regulate the generation of ROS and NO metabolites, as well as the expression of inflammatory and mesenchymal molecules.

**Materials and methods**

Extended information is available in Supplementary materials and methods

**Cell culture, stimulation and pharmacological inhibition**

HUVEC were maintained in endothelial cell culture medium and were used for experiments at passage 6 and 7. Confluent cells were treated for 48 h, with or without 5 or 10 ng/ml citric acid activated-TGF-β1 (Peprotech, USA) in RPMI 1640 basal medium, supplemented with 20% heat-inactivated foetal bovine serum, 1% penicillin-streptomycin, 2 mM L-glutamine and 5 U/ml heparin. For the pharmacological inhibition, cells were treated with appropriate
Shear stress counteracts effects of oxidative stress & TGF-β inhibitors for 48 h in desired media. Cells treated with equal volume of dimethyl sulfoxide (DMSO) served as vehicle control.

**Shear stress experiments**
Confluent cells on flow channel, μ-Slide I Luer (Ibidi, Germany) were exposed to laminar flow with 20 dyn/cm² of shear stress for 48 h in desired media. An Ibidi Pump System was employed for generation of flow.

**Retroviral transduction**
The retroviral construct in pBABE-puro for stable expression of constitutively active rat MEK5-α1 (MEK5D) was kindly provided by Professor Dr. Marc Schmidt (University of Wuerzburg, Wuerzburg, Germany). Retroviral transduction was performed as previously described. 14

**ROS measurement**
Fluorescent result from oxidation of 2’,7’-dichloroflorescin diacetate (DCFDA) by hydrogen peroxide (H₂O₂), peroxynitrite (ONOO-), hydroxyl radicals (•OH) and superoxide anions (O₂⁻) were measured as read-out of intracellular ROS formation. 43

**Quantification of nitrite, nitrate and nitroso compounds**
The concentrations of nitrite and nitrate in cell culture supernatants were determined using dedicated analysis system (ENO-20 with autosampler, EiCom, Japan), as previously described. 44 The level of nitroso compounds in aliquots of the same supernatants was quantified by gas-phase chemiluminescence reaction with ozone. 45

**RNA isolation and quantitative RT-PCR**
RNA was isolated according to the manufacturer’s protocol. Primer sequence for detection of amplimers of interest was presented in Supplementary Table S1.

**Immunofluorescent staining**
Treated cells were fixed at room temperature with 2% paraformaldehyde for 15 min and subjected to immunostaining with mouse anti-human ICAM-1 antibody (hybridoma supernatant; Hu5/3; kindly provided by Professor Dr. Michael A. Gimbrone Jr, Harvard Medical School, Boston, MA, USA) or rabbit anti-human smooth muscle 22α (SM22α) antibody (1:200; Abcam, Cambridge, UK; ab14106).
Western blotting

An Odyssey Western Blotting System (Li-COR Biosciences, USA) was employed. Detection of protein of interest was achieved with antibodies against human VCAM-1 (1:100; Santa Cruz Biotechnology, USA; sc-8304), ERK5 (1:500; Upstate Cell Signaling Solutions, USA; #07-039), KLF4 (1:500; Santa Cruz Biotechnology; sc-20691), KLF2 (1:500; Santa Cruz Biotechnology; sc-28675), p-SMAD2 (Ser465/467; 1:200; Cell Signaling Technology, USA; #3108), SMAD2/3 (1:200; Cell Signaling Technology; #3102) and GAPDH (1:2000; Abcam, UK; ab9484).

Sandwich enzyme-linked immunosorbent assay (ELISA)

Concentration of IL-8 in culture media was quantified with Human IL-8 ELISA MAX™ Standard Sets (BioLegend Inc, USA) according to the manufacturer’s protocol. Concentration of IL-8 in each media was normalized to their respective numbers of cell under each experimental condition. Data are presented as fold change in IL-8 concentration (pg/ml) relative to their respective experimental controls.

Statistical Analysis

All experimental data were obtained from two to seven independent experiments with duplicates or triplicates. All data are presented as mean±standard error of the mean (SEM). Statistical analyses were performed with GraphPad Prism (Version 6.01; GraphPad Software, Inc., USA). For two-group comparisons, two-tailed ratio paired t test was performed. For multiple group comparisons, one-way analysis of variance (ANOVA) followed by Dunnett’s or Sidak’s post-test were carried out. For multiple categorical group comparisons, two-way ANOVA followed by Sidak’s post-test were executed. All statistical analyses were performed at the 95% of confidence interval. Differences between means were considered significant when probabilities ($P$) were less than 0.05.

Results

TGF-β1 aggravates the pro-inflammatory effects of oxidative stress

Bovine brain extract contains endothelial cell growth factors, particularly fibroblast growth factor (FGF) that counteracts a severe form of dysfunction, i.e. EndMT. TGF-β1 is an inducer of EndMT for HUVEC. Interestingly, studies about the combined effects of bovine brain extract omission (referred to as growth factors in the rest of the text) and TGF-β1 on oxidative stress and inflammation, as well as their association with EndMT are scarce. Therefore, we investigated the generation of ROS and NO metabolites, as well as the
expression of inflammatory and mesenchymal molecules by HUVEC that cultured in medium deprived of growth factor and supplemented with TGF-β1. Unstimulated HUVEC appeared as a confluent monolayer with densely packed cobblestone-like cells (Figure 1A, left). However, HUVEC treated with medium deprived of growth factor irrespective of the presence of additional TGF-β1 had poor viability and a disrupted monolayer. These cells were hypertrophic with either an elongated (black arrows) or a broad spreading morphology (white arrowheads) (Figure 1A, middle & right). Growth factor deprivation led to a 1.6-fold ($P<0.0001$) increase of intracellular ROS, but stimulation with TGF-β1 did not result in higher ROS induction. Similarly, growth factor deprivation resulted in a 2.2-fold ($P<0.0001$) increase in the formation of NO metabolites, whereas TGF-β1 had no added effect in this regard (Figure 1B). These results show that growth factor deprivation was a potent inducer of oxidative stress in HUVEC.

Growth factor deprivation increased the expression of SELE (10.3-fold; $P<0.01$), ICAM1 (14.5-fold; $P<0.0001$), VCAM1 (50-fold; $P<0.0001$), CXCL8 (7.7-fold; $P<0.001$), CCL2 (10.7-fold; $P<0.0001$), CDH5 (1.4-fold; $P<0.01$), VWF (1.7-fold; $P<0.05$), ACTA2 (1.6-fold; $P<0.05$), TAGLN (13.6-fold; $P<0.01$) and CNN1 (3.2-fold; $P<0.0001$). In contrast, the expression of THBD decreased 1.8-fold ($P<0.01$) by growth factor deprivation. In comparison with growth factor deprivation, TGF-β1 caused an additional increase of CXCL8 ($P<0.01$) expression, which was accompanied by a further downregulation of CDH5 ($P<0.05$), PECAM1 ($P<0.001$) and NOS3 ($P=0.1346$; Figure 1C). Growth factor deprivation and/or TGF-β1 did not alter the expression of TNFA and IL1B. The expression of IL6 increased 1.7-fold ($P<0.05$) under stimulation with TGF-β1 (Supplementary Figure S1). Growth factor deprivation enhanced the protein expression of ICAM-1 2.7-fold ($P<0.0001$). However, there was no added effect of TGF-β1 on the expression of ICAM-1 (Figure 1D). Of note, growth factor deprivation did not alter the expression VCAM-1, while TGF-β1 caused a 6-fold ($P<0.01$) upregulation (Figure 1E). TGF-β1 synergised the effect of growth factor deprivation in induction of IL-8 secretion ($P<0.05$). The combined effects of growth factor deprivation and TGF-β1 upregulated IL-8 secretion 5.4-fold ($P<0.001$; Figure 1F). These data indicate TGF-β1 aggravated the pro-inflammatory effects of oxidative stress. Of note, the pro-inflammatory phenotype of endothelial cells was endowed with the feature of EndMT.

**Shear stress preserves phenotype of endothelial cells**

To assess the regulation of endothelial phenotype by shear stress, we performed *in vitro* studies on HUVEC exposed to laminar flow at a magnitude of 20 dyn/cm² shear stress. Shear stress induced alignment of HUVEC parallel to the direction of flow (from *left* to *right* as indicated by white arrows; Supplementary Figure S2A). Exposure of HUVEC to shear stress suppressed
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The expression of **SELE**, **VCAM1**, **CXCL8** and **CCL2** by 3.3- (P<0.01), 1.4- (P<0.05), 19.2- (P<0.0001) and 23.8- (P<0.0001) fold, respectively. In contrast, shear stress led to an upregulation of **ICAM1** (2.3-fold; P<0.001), **VWF** (1.5-fold; P<0.05), **THBD** (11.3-fold; P<0.0001), **NOS3** (6.7-fold; P<0.0001) and **CNN1** (4-fold; P<0.01; Supplementary Figure S2B). In agreement with the gene expression data, shear stress upregulated the protein expression of ICAM-1. TGF-β1 had insignificant effect on the elevation of ICAM-1 expression (n=3). Scale bar represents 50 μm. The combined effect of growth factor deprivation and TGF-β1 accentuated (E) the expression of VCAM-1 (n=3) and (F) the secretion of IL-8 (n=3). *P<0.05, **P<0.01, ***P<0.001 & ****P<0.0001.

Oxidative stress and TGF-β1 did not alter the response of HUVEC to shear stress, as observed by the alignment of cells parallel to the direction of flow (from left to right as indicated by white arrows; Figure 2A). However, under these conditions, HUVEC appeared more hypertrophic and less arranged as compared to controls in complete medium. In comparison with the static control, sheared HUVEC had a 6- (P<0.01), 4- (P<0.0001), 11.5- (P<0.0001), 94- (P<0.0001) and 42- (P<0.0001) fold downregulation of **SELE**, **ICAM1**, **VCAM1**, **CXCL8** and **CCL2**, respectively. Notably, shear stress repressed the upregulation of **CXCL8** and **CCL2** below the baseline that is defined by unstimulated static control. Shear stress reduced the expression of **CDH5** by 1.5-fold (P<0.01), but augmented the expression of **PECAM1**, **THBD** and **NOS3** 1.6- (P<0.001), 30- (P<0.0001) and 7.5- (P<0.0001) fold, respectively. The expression of **TAGLN** induced by oxidative stress and TGF-β1 was 1.7-fold (P<0.05) lower under shear. Shear stress did not alter the expression of **VWF**, **ACTA2** and **CNN1** under oxidative stress and TGF-β1 stimulation (Figure 2B). Shear stress enhanced the protein expression of ICAM-1 (data not shown), but suppressed the upregulation of VCAM-1 (P<0.001; Figure 2C) and IL-8 (P<0.01; Figure 2D) to basal levels. Evidently, shear stress attenuated

Figure 1. TGF-β1 aggravates the pro-inflammatory effects of growth factor deprivation that induces oxidative stress. (A) Confluent monolayer of HUVEC was disrupted after 48 h of treatment with media deprived of growth factors and supplemented with TGF-β1. Black arrow indicates cells with an elongated, spindle-shape morphology. White arrowhead indicates cells with an enlarged, broad spreading morphology. Representative phase contrast micrographs at 25x original magnifications are shown. Scale bar represents 200 μm. (B) Growth factor deprivation induced the formation of intracellular ROS and NO metabolites (n=3). TGF-β1 had negligible effects on the production of ROS and NO (n=3). (C) Growth factor deprivation induced the gene expression of adhesion molecules (**SELE**, **ICAM1** and **VCAM1**), chemoattractants (**CXCL8** and **CCL2**) and mesenchymal markers (**ACTA2**, **TAGLN** and **CNN1**) as compared with unstimulated condition (shown as a dotted line). TGF-β1 augmented the upregulation of **CXCL8** and downregulation of **CDH5**, **PECAM1** and **NOS3** (n=3). (D) Growth factor deprivation induced the protein expression of ICAM-1. TGF-β1 had insignificant effect on the elevation of ICAM-1 expression (n=3). Scale bar represents 50 μm. The combined effect of growth factor deprivation and TGF-β1 accentuated (E) the expression of VCAM-1 (n=3) and (F) the secretion of IL-8 (n=3). *P<0.05, **P<0.01, ***P<0.001 & ****P<0.0001.
Figure 2. Shear stress preserves phenotype of HUVEC. (A) Morphology of HUVEC under static condition and exposure to shear stress in growth factor-deprived, TGF-β1-supplemented medium. White arrow indicates the direction of flow. Representative phase contrast micrographs at 25x original magnifications are shown. Scale bar represents 200 μm. (B) Shear stress downregulated the elevated expression of adhesion molecules, chemotactants and mesenchymal marker, particularly TAGLN. Growth factor deprivation and TGF-β1 did not alter the effects of shear stress on upregulating the expression of THBD and NOS3 (n=3). (C & D) Shear stress downregulated the increased expression of VCAM-1 (n=7) and IL-8 (n=4) resulted from growth factors deprivation and TGF-β1 stimulation. Data are presented relative to the unstimulated static condition (shown as a dotted line). *P<0.05, **P<0.01, ***P<0.001 & ****P<0.0001.
the combined pro-inflammatory effects of oxidative stress and TGF-β1 and antagonised the unfavourable alteration of endothelial phenotype.

**Activation of ERK5 restores redox balance, but does not repress the effects of TGF-β1**

We were intrigued by the question as to how the combined effects of oxidative stress and TGF-β1 may affect the ERK5 pathway in regulating of endothelial phenotype. To address this, we examined the effects of growth factor deprivation and TGF-β1 on HUVEC transduced with MEK5D under static culture. MEK5D is a constitutively active mutant of MEK5 that mediates sustained activation of the ERK5 pathway.\(^{14}\) After 48 h treatment with growth factor deprivation and TGF-β1, vector controls showed poorer viability than MEK5D-transduced HUVEC and did not form a confluent monolayer (Figure 3A). Constitutive activation of ERK5 repressed the formation of both ROS and NO metabolites to about the same magnitude (1.9-fold, \(P<0.05\); Figure 3B). TGF-β1 did not alter the effects of ERK5 on repressing the generation of ROS and NO metabolites. These data suggest that activation of the ERK5 pathway counteracts oxidative stress.

Sustained activation of ERK5 enhanced the expression of ICAM1 and VCAM1 by a factor of 4- (\(P<0.0001\)) and 4.9- (\(P<0.0001\)), respectively. Notably, TGF-β1 synergised the ERK5-mediated upregulation of ICAM1 (\(P<0.0001\)) and VCAM1 (\(P<0.0001\)). In MEK5D-transduced HUVEC, the expression of NOS3 was increased by 4-fold (\(P<0.0001\)) despite the stimulation of TGF-β1. The ERK5 pathway did not influence the expression of TAGLN under unstimulated condition. However, there was a 4-fold (\(P<0.0001\)) increased expression of TAGLN in MEK5D-transduced HUVEC under stimulation with TGF-β1, indicating expression of TAGLN was driven predominantly by TGF-β1 (Figure 3C). In spite of the enhanced transcript expression, TGF-β1 had negligible effect on protein expression of ICAM-1 in MEK5D-transduced HUVEC (Figure 3D), whereas sustained activation of ERK5 induced the expression of VCAM-1 by a factor of 5 (\(P<0.05\)). Interestingly, VCAM-1 expression was induced 17-fold (\(P<0.05\)) when the MEK5D-transduced HUVEC were treated with TGF-β1 (Figure 3E). The increased secretion of IL-8 upon stimulation with TGF-β1 was strongly inhibited (14.6-fold; \(P<0.001\)) when MEK5D was stably expressed (Figure 3F). Together, our data indicate that activation of the ERK5 pathway failed to repress the TGF-β1-induced alterations in endothelial phenotype.
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Shear stress antagonises the activation of canonical TGF-β signalling, independent of ERK5

Under stimulation with TGF-β1, shear stress repressed, whereas ERK5 signalling augmented the expression of TAGLN and VCAM-1. These differences prompted us to dissect the underlying mechanisms. In spite of the TGF-β1 stimulation, both sheared and MEK5D-transduced HUVEC showed increased ERK5 phosphorylation, as well as enhanced KLF2 and KLF4 expression (Figure 4A). Boon et al.26 elucidated KLF2 attenuates canonical TGF-β signalling through reduction of SMAD2 phosphorylation and inhibition of SMAD3/4 transcriptional activity. We sought to investigate whether shear stress which induces KLF2 expression via the ERK5 signalling axis, downregulates the canonical TGF-β signalling. In the present study, phosphorylation of SMAD2 was used to validate the activation of canonical TGF-β signalling. The level of SMAD2 phosphorylation in complete medium was not altered by shear stress. TGF-β1 stimulation induced SMAD2 phosphorylation ($P=0.1504$), which was repressed approximately 1.5-fold ($P<0.05$) by shear. Of note, this repression was not caused by the reduced expression of total SMAD2 (Figure 4B). Activation of ERK5 signalling under static conditions did not suppress the phosphorylation of SMAD2. Interestingly, upon stimulation with TGF-β1, the MEK5D-transduced HUVEC showed a 2.3-fold ($P<0.05$) higher phosphorylation of SMAD2 than the vector controls, implying that the ERK5 pathway synergises with the activation of TGF-β signalling. MEK5D did not alter the expression of total SMAD2 (Figure 4C). These results demonstrate that shear stress suppresses, whereas ERK5 signalling enhances the activation of canonical TGF-β signalling.
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Shear stress induced the expression of **SMAD6** and **SMAD7** 2.4- (**P**<0.0001) and 4.1- (**P**<0.0001) fold, respectively. Shear-induced **SMAD6** and **SMAD7** expression was not altered by the presence or absence of TGF-β1 (Figure 4D). In concordance with shear stress, activation of ERK5 under static conditions enhanced the expression of **SMAD6** and **SMAD7** 2.4- (**P**<0.05) and 4.5- (**P**<0.001) fold, respectively. Intriguingly, stimulation with TGF-β1 augmented the upregulation of **SMAD6** (**P**<0.05) and **SMAD7** (**P**<0.05) in MEK5D-transduced HUVEC (Figure 4E), supporting the notion that ERK5 signalling enhances the activation of canonical TGF-β signalling. Shear stress did not change the expression of **SMAD4** (Figure 4F). Activation of ERK5 slightly suppressed the expression of **SMAD4** (**P**<0.001; Figure 4G). Stimulation with TGF-β1 led to a modest decrease in the expression of **SMAD4** (**P**<0.05) in static culture, under shear stress (Figure 4F) and upon activation of the ERK5 pathway (Figure 4G). These data show that both shear stress and activation of ERK5 induced the expression of inhibitory SMADs (I-SMADs), *i.e.* **SMAD6** and **SMAD7** potently, yet the effects of shear stress and ERK5 activation on the common-mediator SMAD (co-SMAD), *i.e.* **SMAD4** were relatively less robust.

**Inhibition of canonical TGF-β signalling suppresses oxidative stress and **TAGLN** expression, but does not influence the expression of ICAM-1, VCAM-1 and IL-8**

Since shear stress represses the activation of canonical TGF-β signalling, we, sought to inhibit the SMAD pathway with the ALK5 inhibitor, SB431542 to explore its effects on redox balance and cellular phenotype. Inhibition of ALK5 signalling reduced the formation of intracellular ROS and NO metabolites 1.3- (**P**<0.01) and 2.2- (**P**<0.01) fold, respectively (Figure 5A). In addition to that, we also observed a downregulation of **ICAM1**, **CXCL8**, **CCL2** and **TAGLN** by 1.9- (**P**<0.01), 1.8- (**P**<0.001), 1.9- (**P**<0.01) and 3.5- (**P**<0.0001) fold, respectively. ALK5 signalling had no effect on the expression of **VCAM1** and **IL-8**.
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Figure 5. Inhibition of canonical TGF-β signalling suppresses oxidative stress and TAGLN expression, but does not influence the expression of ICAM-1, VCAM-1 and IL-8. (A) Attenuation of ALK5 kinase activity reduced the level of intracellular ROS and NO metabolites (n=3). (B) ALK5 inhibition antagonised the induced expression of ICAM1, CXCL8, CCL2 and TAGLN, but not VCAM1 and SELE. (C, D & E) ALK5 inhibition did not repress the induced protein level of ICAM-1, VCAM-1 and IL-8 (n=3). (F & G) The enhanced expression of ICAM-1 and VCAM-1 in MEK5D-transduced HUVEC remained unaltered despite the inhibition of ALK5 (n=3). Scale bar represents 50 μm. (H) Representative western blotting of inhibition of SMAD2 phosphorylation by SB431542. *P<0.05, **P<0.01, ***P<0.001 & ****P<0.0001.

SELE (Figure 5B). Downregulation (4.5-fold; P<0.0001) of TGF-β1-induced TAGLN expression was evident in the MEK5D-transduced HUVEC treated with SB431542 (Supplementary Figure S3A). Inhibition of the ALK5 signalling had no effects on the induced expression of ICAM-1 (Figure 5C), VCAM-1 (Figure 5D) and IL-8 (Figure 5E) under TGF-β1 stimulation. Similarly, the expression of ICAM-1 (Figure 5F) and VCAM-1 (Figure 5G) in MEK5D HUVEC were unaffected by the inhibition of ALK5. Activation of SMAD2 was strongly inhibited by SB431542 in both wild-type and MEK5D-transduced HUVEC (Figure 5H). This data implicates a role for ALK5 signalling in redox balance and TAGLN expression, but not in the expression of inflammatory molecules.

Inhibition of TAK1 pathway or mitochondrial ROS production suppresses the upregulation of ICAM-1, VCAM-1, IL-8 and SM22α

Since inhibition of ALK5 signalling did not suppress ROS-induced expression of ICAM-1, VCAM-1 and IL-8, we hypothesised that shear stress inhibits the expression of these molecules by regulating either non-canonical TAK1 signalling or redox balance. The TAK1 inhibitor, 5z-7-oxozeaenol and the mitochondrial ROS inhibitor, YCG063 reduced the formation of intracellular ROS 1.2- (P<0.05) and 1.4- (P<0.0001) fold, respectively. Inhibition of TAK1 led to a 2-fold (P<0.01) reduction in the formation of NO metabolites. While inhibition of mitochondrial ROS production had little to no effect on the production of nitrite and nitrate, the production of nitroso compounds was enhanced by more than 2 orders of magnitude (137-fold; P<0.01; Figure 6A). 5Z-7-oxozeaenol suppressed the induced transcript level of VCAM1, SELE, ICAM1, CXCL8, CCL2 and TAGLN by 15.3- (P<0.01), 5.8- (P<0.0001), 4.5- (P<0.0001), 21- (P<0.0001), 2.6- (P<0.0001) and 2.4- (P<0.0001) fold, respectively. Similarly, YCG063 also downregulated the induced expression of VCAMI, SELE, ICAM1, CXCL8, CCL2 and TAGLN by 33.9- (P<0.01), 2.6- (P<0.0001), 3.9- (P<0.0001), 12.7- (P<0.0001), 3.5- (P<0.0001) and 8.6- (P<0.0001) fold, respectively. NOS3 expression was unaffected by 5z-7-oxozeaenol and YCG063 (Figure 6B). 5Z-7-oxozeaenol and YCG063 repressed the increased expression of TAGLN in MEK5D-transduced HUVEC by 4.2-
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Figure 6. Suppression of either TAK1 activation or mitochondrial ROS production represses the upregulation of adhesion molecules, chemoattractants and mesenchymal marker. (A) Both 5z-7-oxozeaenol and YCG063 reduced the production of intracellular ROS (n=3). 5z-7-oxozeaenol reduced the level of NO metabolites. YCG063 had little to no effect on the formation of nitrite and nitrate, but it induced the formation nitroso compounds potently (n=3). (B, C, D & E) Inhibition of TAK1 or suppression of mitochondrial ROS generation counteracted the induced expression of SELE, ICAM1, VCAM1, CXCL8, CCL2 and TAGLN, as well as ICAM-1, VCAM-1 and IL-8 (n=3). (F & G) The induced expression of ICAM-1 and VCAM-1 in MEK5D HUVEC were repressed by either 5z-7-oxozeaenol or YCG063 (n=3). Scale bar represents 50 μm. *P<0.05, **P<0.01, ***P<0.001 & ****P<0.0001.

(P<0.001) and 6.4- (P<0.0001) fold, respectively (Supplementary Figure S3B). Upon treatment with 5z-7-oxozeaenol and YCG063, the upregulation of SM22α decreased 2.3- (P<0.001) and 1.8- (P<0.01) fold (Supplementary Figure S4), respectively, which was accompanied by a 3-fold downregulation of ICAM-1 (P<0.05; Figure 6C). The increased level of VCAM-1 was respectively downregulated by 14.6- (P<0.01) and 113.2- (P<0.01) fold in response to the treatment of 5z-7-oxozeaenol and YCG063 (Figure 6D). Inhibition of TAK1 or mitochondrial ROS formation resulted in a 1.9- (P<0.01) and 4.3- (P<0.01) fold reduction in the level of IL-8 release (Figure 6E). Under the treatment of 5z-7-oxozeaenol or YCG063, the ICAM-1 expression of MEK5D-transduced HUVEC were repressed almost 2-fold (P<0.05) (Figure 6F). VCAM-1 expression of MEK5D-transduced HUVEC was respectively downregulated by 3.4- (P<0.01) and 6.1- (P<0.001) fold in response to the treatment of 5z-7-oxozeaenol and YCG063 (Figure 6G). Taken together, these data show that TAK1 and/or generation of mitochondrial ROS contribute to oxidative stress, with an upregulation of inflammatory and mesenchymal molecules.

Inhibition of p38 MAPK and NFκB pathways, but not JNK pathway suppress pro-inflammatory endothelial phenotype

Finally, we sought to dissect the signalling cascades that act downstream of the TAK1 pathway in attenuation of oxidative stress and suppression of endothelial dysfunction. We, therefore treated HUVEC with p38 MAPK inhibitor (SB202190), IKKβ inhibitor (SC514) and JNK inhibitor (SP600125) under stimulation of TGF-β1 in growth factors-deprived medium. SB202190 decreased the induced formation of intracellular ROS 1.3-fold (P<0.01). SC514 had no effect on the formation of ROS. Both SB202190 and SC514 reduced the formation of NO metabolites by 2.1- (P<0.01) and 1.7- (P<0.05) fold, respectively (Figure 7A). SB202190 decreased the induced transcript level of VCAMI, SELE, ICAMI, CXCL8, CCL2 and TAGLN 18.1- (P<0.0001), 5.6- (P<0.05), 10.2- (P<0.0001), 8.7- (P<0.0001), 7.4- (P<0.0001) and 3- (P<0.0001)
Figure 7. Inhibition of either p38 MAPK or NFκB pathway, but not JNK pathway antagonises the upregulation of adhesion molecules, chemoattractants and mesenchymal marker. (A) P38 MAPK inhibition reduced the production of intracellular ROS, whereas inhibition of either p38 MAPK or NFκB pathway decreased the level of NO metabolites (n=3). (B) Inhibition of either p38 MAPK or NFκB pathway suppressed the induced expression of SELE, ICAM1, VCAM1, CXCL8, CCL2 and TAGLN (n=3). (C) Inhibition of JNK tended to suppress the upregulation of SELE and stimulated the expression of TAGLN and NOS3. It has no effect on the expression of ICAM1, VCAM1, CXCL8 and CCL2 (n=3). *P<0.05, **P<0.01, ***P<0.001 & ****P<0.0001.
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fold, respectively. Similarly, SC514 also decreased the induced expression of VCAM1, SELE, ICAM1, CXCL8, CCL2 and TAGLN 38- (P<0.0001), 21.5- (P<0.01), 13- (P<0.0001), 20- (P<0.0001), 26- (P<0.0001) and 1.9- (P<0.01) fold, respectively. NOS3 expression was unaffected by SB202190 and SC514 (Figure 7B). SB202190 and SC514 repressed the increased expression of TAK1 pathway preferentially signals to p38 MAPK and/or NFkB pathways upon stimulation of TGF-β1 for induction of pro-inflammatory endothelial phenotype.

Discussion

In this study, we dissected the interplay of different components of the mechanotransduction signalling network that have been implicated in the suppression of the pro-inflammatory endothelial phenotype by high shear stress. Our *in vitro* data reveal that high shear stress counteracts the pro-inflammatory effects of TGF-β1 by suppressing the TAK1 pathway, in a manner independent of ERK5 signalling, redox balance and ALK5 kinase activity. Notably, shear stress-activated ERK5 signalling only restored the redox balance of endothelial cells, but failed to antagonise the pro-inflammatory effects of TGF-β1. We further show that shear stress abates the kinase activity of ALK5. However, ERK5 activation in static culture led to enhanced ALK5 activity, indicating that suppression of canonical TGF-β signalling by shear stress is independent of the ERK5 pathway (Figure 8). Of note, suppression of canonical TGF-β signalling by shear stress might lead to inhibition of EndMT, but could not rescue the endothelial cells from a pro-inflammatory phenotype.

Oxidative stress can reduce NO bioavailability following reaction of NO with superoxide, a reaction that also gives rise to formation of the potent pro-oxidant, peroxynitrite. Peroxynitrite and other ROS can alter endothelial phenotype by disrupting NO and redox signalling, as well as by inducing the activation of NFκB, JNK and p38 MAPK. Although short-term treatment with TGF-β1 induces ROS generation, the influence of TGF-β1 on HUVEC redox status appear to be minor when compared to long-term growth factor deprivation. Our data demonstrate that TGF-β1 aggravates the pro-inflammatory effects of oxidative stress. This occurs via the non-canonical TGF-β pathway, as the expression of inflammatory molecules was unaffected by inhibition of the canonical SMAD pathway. Consistent with previous studies, we showed that activation of ERK5 repressed the induction of inflammatory molecules,
particularly SELE, CCL2 and IL-8. Intriguingly, ERK5 signalling had negligible inhibitory effects on the VCAM-1 expression, for the transcription of VCAM1 was governed by the TAK1 pathway upon stimulation of TGF-β1. Of note, expression of VCAM-1 decreased when the TAK1 signalling axes was inhibited. A selective inhibition of either NFκB or p38 MAPK, but not JNK, robustly downregulated the expression of inflammatory molecules. This coincides with earlier findings that TAK1 requires NFκB and p38 MAPK to elicit its downstream effects in endothelial cells. In addition, our data imply that shear stress downregulates oxidative stress-induced NFκB and p38 MAPK activation by attenuating the generation of mitochondrial ROS. Activation of TAK1 depends predominantly on the affinity of ALK5 to TβRI and stimulation of TβRII by TGF-β1, which suggests that shear stress suppresses the activation of TAK1 by interfering either with the affinity of ALK5 to TβRI or the binding of TGF-β1 to TβRII. Notably, shear stress can repress oxidative stress-induced inflammation by restoring the redox balance, yet the pro-inflammatory effects of TGF-β1 are predominantly counteracted via the TAK1 pathway.

We reported earlier that the ERK5 pathway attenuated oxidative stress and augmented NOS3 expression. AMP-activated protein kinase (AMPK) mediates shear stress-induced ERK5 signalling, while increasing NO bioavailability and reducing oxidative stress. This mechanistic evidence links shear stress with the ERK5 pathway in redox homeostasis. Interestingly, Xie et al. identified TAK1 as an upstream activator for AMPK in vivo and in vitro, which implies that TAK1 may reduce oxidative stress via AMPK activation. Intriguingly, our data show that inhibition of TAK1 attenuated oxidative stress and this finding is in accordance with a similar observation in vascular smooth muscle cells. Yamada et al. and Huot et al. reported oxidative stress activates p38 MAPK and vice versa in endothelial cells. We demonstrated that inhibition of the p38 MAPK pathway reduced oxidative stress too. Therefore, p38 MAPK might act as a downstream modulator for TAK1 in inducing ROS production. Of note, suppression of TAK1 and p38 MAPK did not induce NOS3 expression in the same way as activation of the ERK5 pathway, hence TAK1 and p38 MAPK might be the mechanotransducers secondary to ERK5 in maintenance of redox homeostasis. On the other hand, Jiang et al. revealed activation of ERK5 by shear stress leads to NFκB activation in osteoblasts. Therefore, we suggest shear stress attenuates oxidative stress predominantly via activation of the ERK5 pathway, but antagonises pro-inflammatory endothelial phenotype by suppressing TAK1-mediated p38 MAPK and NFκB pathways.

The amino acid sequence of SMAD3 is 91% identical to those of SMAD2. These R-SMADs also appear to have the same functions. SMAD6 is structurally very similar to SMAD7. Intriguingly, SMAD6 only inhibits the phosphorylation of SMAD2, whereas SMAD7 can inhibit the phosphorylation of both SMAD2 and SMAD3. SMAD6 and SMAD7 which are induced upon
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stimulation of TGF-β ligand, are negative feedback regulators of the TGF-β signalling. KLF2 via upregulation of SMAD7, represses phosphorylation of SMAD2 and limits transcriptional activity of SMAD3/4. Notably, SMAD2 phosphorylation was not attenuated in our MEK5D-transduced HUVEC, despite the enhanced expression of KLF2, SMAD6 and SMAD7. SMAD6 and SMAD7 inhibit phosphorylation of SMAD2 by binding stably to the ALK5. It is unlikely that activation of ERK5 changes ALK5 conformation such that it compromises inhibitor affinity, because SMAD2 phosphorylation was abolished under treatment of SB431542. Alternatively, SMAD6 and SMAD7 can repress TGF-β signalling by preventing SMAD2 from coupling with SMAD4 and inhibiting nuclear accumulation of the complex. The overexpression of SMAD2 target gene, TAGLN under stimulation with TGF-β1 clarifies the transcriptional activity of p-SMAD2 in MEK5D-transduced HUVEC was conserved, supporting the notion that upregulation of SMAD6 and SMAD7 by ERK5 pathway was not associated with the downregulation of ALK5 signalling. Additional mechanisms might be involved instead. For example, SMAD6 and SMAD7 proteins in MEK5D-transduced cells may be malfunctioning or have been degraded due to SUMO(small ubiquitin-like modifier)ylation and ubiquitylation under stimulation with TGF-β1. Alternatively, the post-transcriptional processes of these inhibitory SMADs may be affected by microRNAs. On the contrary, shear stress-induced SMAD6 and SMAD7 expression was correlated with the downregulation of SMAD2 phosphorylation. The inhibitory effect of shear stress on SMAD4 expression was relatively less robust than those on SMAD6 and SMAD7, indicating that shear stress downregulates ALK5 signalling predominantly by augmenting the expression of inhibitory SMADs. Egorova et al. reported shear stress activated-ALK5 signalling mediates the induction of KLF2 in embryonic endothelial cells. Walshe et al. revealed that shear stress upregulates the expression of TGF-β3 in HUVEC and lead to higher expression of KLF2 via ALK5 signal transduction. Our findings, on the other hand, elucidate shear stress attenuates TGF-β1-induced ALK5 activation via upregulation of I-SMADs, independent of the ERK5 pathway and redox balance.

Indeed, regulation of endothelial phenotype by shear stress involves an intricate crosstalk of multiple signalling axes, rather than an alteration in distinct individual pathways (Figure 8). Our previous findings elucidated shear stress mediates EndMT through ERK5 pathway. Here, we showed that parallel to signalling through ERK5, which safeguards redox balance, shear stress antagonises the pro-inflammatory effects of TGF-β1 by suppressing the TAK1 pathway. TGF-β1-driven EndMT is potentially alleviated by attenuation of ALK5 signalling. Untangling the interplay of various mechanotransduction signalling cascades may provide new avenues for the treatment of cardiovascular disorders and drug development.
Figure 8. Proposed mechanosignalling crosstalk for regulation of endothelial phenotype by shear stress under oxidative stress and stimulation of TGF-β. ROS derived from mitochondria tilts the intracellular redox balance by eliminating NO. Superoxide anions (O2•⁻) can react with NO to yield peroxynitrite (ONOO⁻) that brings about eNOS uncoupling. ROS activates p38 MAPK and NFκB signalling cascades which induce the expression of inflammatory and mesenchymal molecules. Shear stress represses the expression of inflammatory and mesenchymal molecules by attenuating the generation of mitochondrial ROS and increasing the bioavailability of NO that might occur via the ERK5 and AMPK pathway. TGF-β stimulates the activation of p38 MAPK and NFκB signalling cascades too. Shear stress represses the TGF-β-induced p38 MAPK and NFκB signalling cascade via inactivation of TAK1, in a manner independent of ERK5, redox balance and ALK5 kinase activity. Shear stress-induced activation of ERK5 signalling via the induction of anti-inflammatory transcription factors, KLF2 and KLF4, enhances the expression of NOS3, SMAD6 and SMAD7, while suppresses the expression of SELE, CXCL8 and CCL2. Shear stress attenuates the ALK5/SMAD pathway via the upregulation of inhibitory SMADs, i.e. SMAD6 and SMAD7, by which the TGF-β-induced TAGLN expression is diminished. There might be a positive feedback loop between generation of ROS and activation of ALK5, TAK1 and p38 MAPK.
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References


3

Supplementary materials and methods

**Cell culture, stimulation and pharmacological inhibition**

Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (MD, USA) or provided by The Laboratory of Endothelial Biomedicine & Vascular Drug Targeting, Department of Pathology and Medical Biology, University Medical Center Groningen, The Netherlands as described before.\(^1\) Cells were maintained on gelatin-coated flasks (0.1% gelatin in distilled water) in endothelial cell culture medium, composed of, RPMI 1640 basal medium (Lonza, Verviers, Belgium), supplemented with 20% heat-inactivated foetal bovine serum (Invitrogen/GIBCO, CA, USA), 50 μg/ml bovine brain extract (homemade), 1% penicillin-streptomycin (Sigma-Aldrich, MA, USA), 2 mM L-glutamine (Lonza) and 5 U/mL heparin (Leo Pharma, Ballerup, Denmark). Trypsin (MP Biomedicals, Illkirch, France) in EDTA was used for the detachment of cells. Cells were seeded on fibronectin-coated (1.5 μg/cm\(^2\); Harbor Bio-Products, MA, USA; 2003 & Alfa Aesar, MA, USA; J64560) plates, left to form a confluent monolayer before stimulation. Cells treated with endothelial cell culture medium were harvested as unstimulated control. SB431542 (10 μM; Sigma-Aldrich, MO, USA; S4317), 5z-7-oxozeaenol (10 μM; Sigma-Aldrich; O9890), YCG063 (50 μM; Calbiochem, CA, USA; #557354), SB202190 (10 μM; Sigma-Aldrich; S7067), SC514 (100 μM; Santa Cruz Biotechnology, CA, USA; sc-205504A) and SP600125 (10 μM; Calbiochem; #420128) were used for inhibition of desired signalling pathways. Cells treated with equal volume of dimethyl sulfoxide (DMSO) served as vehicle control. All cell cultures were performed in an incubator at 37°C with 5% CO\(_2\).
Shear stress experiments

Cells were seeded on fibronectin-coated (1.5 μg/cm²) flow channel, µ-Slide I⁰.⁴ Luer (Ibidi, Martinsried, Germany). Confluent cells were exposed to flow in endothelial cell medium or RPMI 1640 basal medium, supplemented with 10 ng/mL TGF-β1, 20% heat-inactivated foetal bovine serum, 1% penicillin-streptomycin, 2 mM L-glutamine and 5 U/ml heparin. Parallel with the shear stress experiments, cells were maintained under static condition as controls. Shear stress experiments were performed in an incubator at 37°C with 5% CO₂.

Retroviral transduction

Vector with desired construct or empty vector was transfected into Phoenix retrovirus producer cells. Phoenix cell culture was expanded in DMEM basal medium, supplemented with 10% heat-inactivated foetal bovine serum, 1% penicillin-streptomycin and 1% L-glutamine. At 50% confluence, medium on Phoenix cells was changed to fresh endothelial cell culture medium. Endothelial cell culture medium containing retroviruses was collected every 24 h, filtered through 0.45 μm filter, supplemented with 6 μg/ml polybrene and transferred to HUVEC cultures for a 24-h-transduction. Retroviral transduction was performed in three consecutive rounds. At 72 h post-transduction, transduced cells were selected for puromycin resistance (2 μg/ml puromycin, 24 h) and expanded on gelatin-coated dishes. Expanded transduced cells were seeded on fibronectin coated dishes for experiments as described for wild type cells.

ROS measurement

Cells were washed with RPMI 1640 basal medium and detached with accutase (Sigma-Aldrich). Detached cells were centrifuged at 4°C in RPMI 1640 basal medium, supplemented with 10% heat-inactivated foetal bovine serum and 1% penicillin-streptomycin. Cell pellets were re-suspended in RPMI 1640 basal medium consisted of 20 μM of 2’,7’-dichloroflorescin diacetate (DCFDA; Sigma-Aldrich; D6883) and incubated for 30 min at 37°C with 5% CO₂. Stained cells were analysed with FACSCalibur™ flow cytometer (BD Biosciences, NJ, USA) by detecting the green fluorescent cells at λmax, 520 nm. Data analysis was performed with Kaluza® Flow Analysis Software version 1.3 (Beckman Coulter Inc, California, USA). Average intensity was obtained by substracting the mean fluorescent intensity (MFI) of unstained samples from stained samples. Data of each experimental condition are presented as fold change in average intensity relative to their respective experimental controls.

Quantification of nitrite, nitrate and nitroso compounds

The measurement of NO is technically challenging in both in vitro and in vivo models due to its low concentration and rapid reactions with various biochemical compounds.² In the presence of oxygen, NO is oxidised rapidly
to nitrite (NO$_2^-$) and nitrate (NO$_3^-$). Alternatively, NO is eliminated by collision with reactive oxygen species, *i.e.* superoxide anions (O$_2^\bullet^-$) to yield oxidant peroxynitrite (ONOO$^-$) which either decomposes to nitrite and nitrate or oxidises and nitrosates other biochemical compounds. Also, NO can react with oxygen to produce reactive nitrogen oxide intermediates, such as nitrogen dioxide (NO$_2$) and dinitrogen trioxide (N$_2$O$_3$). Reactive nitrogen oxide intermediates can oxidise and nitrosate various biochemical compounds and give rise to nitroso compounds (RXNOs), such as N-nitrosamines (RNNOs) and S-nitrosothiol (RSNOs) (Rassaf et al., 2004). NO metabolites, *i.e.* nitrite, nitrate and nitroso compounds are more stable than NO, thereby, are measured as an indirect read-out of NO generation.\textsuperscript{3,4,5} Data of each experimental condition are presented as fold change in concentration of nitrite (μM), nitrate (μM) and nitroso compounds (nM) relative to their respective controls.

**RNA isolation and quantitative RT-PCR**

RNA was isolated with either TRizol reagent (Invitrogen Corp, CA, USA) or RNA-Bee (Bio-Connect, The Netherlands). Subsequently, 1 μg of total RNA was reverse-transcribed using the FirstStrand cDNA synthesis kit (Fermentas UAB, Lithuania) according to the manufacturer’s protocol. The cDNA-equivalent of 5 ng RNA was used for amplification in 384-well microtitre plates in a TaqMan ABI7900HT cycler (Applied Biosystems, CA, USA) in a final reaction volume of 10 μl containing 5μL SYBR Green mix with ROX (Bio-Rad Laboratories, CA, USA or Roche, IN, USA) and 0.5 μl primers mix (Biolegio, Nijmegen, The Netherlands or Sigma-Aldrich). All cDNA samples were amplified in duplicate. Cycle threshold (Ct) values for individual reactions were determined using ABI Prism SDS 2.2 data processing software (Applied Biosystems). The following formulas were employed for calculation of relative fold change for gene expression in different experimental conditions: Ct-values of gene of interest were first normalized against geometric mean of housekeeping genes, *B2M* and *GAPDH* expression by the following equation [ΔCt(GENE) = Ct(GENE) – Ct(geometric mean of B2M and GAPDH)]. Next, relative gene expression levels, ΔΔCt(GENE) were calculated as followed [ΔΔCt(GENE) = ΔCt(GENE) of samples from different experimental condition – average of ΔCt(GENE) of controls]. Lastly, relative fold change for gene expression in samples were calculated as 2$^{−\Delta\Delta C_t(GENE)}$. Data of each experimental condition are presented as fold change in gene expression relative to their respective experimental controls.

**Immunofluorescent staining**

Confluent cells on fibronectin-coated diagnostic slides (VWR International, Amsterdam, The Netherlands) were subjected to treatment of different experimental conditions for 48 h. Fixed cells were rehydrated with PBS for 10 minutes, followed by a 10-min-permeabilisation with 0.5% Triton X-100.
Shear stress counteracts effects of oxidative stress & TGF-β (Sigma Aldrich). To inhibit non-specific antibody binding, cells were blocked with 10% donkey serum for 10 min. After an overnight incubation with the ICAM-1 or smooth muscle 22α (SM22α) (1:200; Abcam, Cambridge, UK; ab14106) antibody in 10% donkey serum at 4°C, cells were incubated with Alexa Fluor® 488-conjugated donkey anti-mouse IgG or Alexa Fluor® 555-conjugated donkey anti-rabbit IgG (1:200; Life Technologies, CA, USA) in PBS containing 3 μm 4’,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) and 10% normal human serum at room temperature for one hour. Next, slides were mounted with Citifluor AP1 (Agar Scientific, Stansted, UK). Stained slides were analysed using TissueFaxs® Zeiss AxioObserver Z1 Microscope System (TissueGnostics, Vienna, Austria). Images were captured with the PCO/Pixelfly II camera (PCO AG, Kelheim, Germany). MFI quantification was performed with TissueQuest fluorescence analysis software (TissueGnostics). At least 500 cells were counted for each experimental condition.

**Western blotting**

Whole cell lysates were prepared in radio-immunoprecipitation assay (RIPA) buffer (Thermo Scientific, IL) supplemented with 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail (both Sigma-Aldrich). Sonicated cell lysates (50 μg/lane) were separated by gel electrophoresis in a 10% denaturing SDS-polyacrylamide gel and subsequently blotted onto nitrocellulose membrane (Hybond-P; Amersham Pharmacia Biotech, England, UK) according to standard protocols. Blots were blocked for one hour in Odyssey Blocking Buffer (Li-COR Biosciences, Nebraska, USA) at room temperature and incubated at 4°C overnight with appropriate antibodies in Odyssey Blocking Buffer, supplemented with 0.1% Tween-20. Subsequently, blots were incubated with secondary antibodies, i.e. goat anti-rabbit conjugated with IRDye700 or goat anti-mouse conjugated with IRDye800 (both 1:10 000; Li-COR Biosciences). The blots were then scanned using an Odyssey Infrared Imaging System (Li-COR Biosciences) for detection of target proteins. The intensity of bands was quantified using ImageJ version 1.47 (National Institute of Health, MD, USA). GAPDH was used as a loading control. Data of each experimental condition are presented as fold change in arbitrary unit relative to their respective experimental controls.
Supplementary Figure S1. The effect of growth factors deprivation and TGF-β1 stimulation on the expression of TNFA, IL1B and IL6. As compared with unstimulated condition (shown as a dotted line), growth factors deprivation had no effect on the gene expression of TNFA, IL1B and IL6, but the combined effect of growth factor deprivation and TGF-β1 up-regulates the expression of IL6 (n=3). *P<0.05.

References


Supplementary Figure S2. Shear stress regulates the phenotype of endothelial cells. (A) HUVEC aligned parallel to the direction of flow (white arrow) after 48 h of exposure to 20 dyn/cm² of laminar shear stress. Representative phase contrast micrographs at 25x original magnifications are shown. Scale bar represents 200 μm. (B) In comparison with the static condition (fold change was shown as a dotted line), shear stress inhibited the expression of SELE and VCAM1, but induces the expression of ICAM1 and the expression of endothelial markers, particularly VWF, THBD and NOS3. Shear stress had no effect on the gene expression of mesenchymal markers, except for CNN1 (n=3). (C) Shear stress suppressed the protein expression of VCAM-1 as compared with the static condition (n=6). (D) Shear stress had no effect on the secretion of IL-8 by HUVEC (n=4). *P<0.05, **P<0.01, ***P<0.001 & ****P<0.0001.

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Supplementary Figure S3. Treatments of MEK5D-transduced HUVEC with (A) SB431542, (B) 5z-7-oxoozenol, YCG063, (C) SB202190 or SC514 in growth factors-deprived TGF-β1 medium suppressed the induced expression of TAGLN (n=3). *P<0.05, **P<0.01, ***P<0.001 & ****P<0.0001.
Supplementary Figure S4. Treatment with 5z-7-oxozeaenol or YCG063 in growth factors-deprived TGF-β1 medium suppressed the induction of SM22α that co-expressed with ICAM-1 in HUVEC (n=3). *P<0.05, **P<0.01 & ****P<0.0001.
### Supplementary table

#### Table S1. Primer sequences of genes

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<td>CTACTCCAGGCTGTACCTCA</td>
<td>tumour necrosis factor α</td>
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<td>(TNF-α)</td>
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<tr>
<td>IL1B F</td>
<td>AAGCTGGAAATTTGAGTGC</td>
<td>interleukin 1B (IL-1B)</td>
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<tr>
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