The Role of Shear-Induced Transforming Growth Factor-? Signaling in the EndotheliumSignificance

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The role of shear-induced TGF-β signaling in the endothelium

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Abstract

Objective—Vascular endothelial cells (ECs) are continuously exposed to blood flow that contributes to the maintenance of vessel structure and function; however, the effect of hemodynamic forces on transforming growth factor-β (TGF-β) signaling in the endothelium is poorly described. We examined the potential role of TGF-β signaling in mediating the protective effects of shear stress on ECs.

Approach and Results—Human umbilical vein endothelial cells (HUVECs) exposed to shear stress were compared to cells grown under static conditions. Signaling through the TGF-β receptor ALK5 was inhibited with SB525334. Cells were examined for morphological changes and harvested for real-time PCR, western blot analysis, apoptosis, proliferation and immunocytochemistry. Shear stress resulted in ALK5-dependent alignment of HUVECs as well as attenuation of apoptosis and proliferation compared to static controls. Shear stress lead to an ALK5-dependent increase in TGF-β3 and Krüppel-like factor 2 (KLF2), phosphorylation of endothelial nitric oxide synthase (eNOS) and NO release. Addition of the NO donor S-nitroso-N-acetylpenicillamine (SNAP) rescued the cells from apoptosis due to ALK5 inhibition under shear stress. Knockdown of TGF-β3, but not TGF-β1, disrupted the HUVEC monolayer and prevented the induction of KLF2 by shear.

Conclusions—Shear stress of HUVECs induces TGF-β3 signaling and subsequent activation of KLF2 and NO, and represents a novel role for TGF-β3 in the maintenance of HUVEC homeostasis in a hemodynamic environment.

Keywords

shear stress; endothelium; TGF-β; KLF2; nitric oxide

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Introduction

Mechanical forces associated with blood flow play an important role in maintaining vessel structure and function. ECs lining the vasculature are continuously exposed to shear stress as a result of fluid forces at the blood/EC interface. Shearing of ECs leads to reorganization of the cytoskeleton, morphological alterations, and production of a variety of substances that act upon the ECs themselves and surrounding cells.[1–3]

Coordinated release of growth factors by cells of the vessel wall plays a critical role in development, stabilization and function of the vasculature. TGF-β is a multifunctional growth factor that is a well-established modulator of vascular cells.[4] Of the three mammalian TGF-β's (TGF-β-1, -2, -3), TGF-β1 and TGF-β3 are found in the vasculature. Structural homology for each isoform among species is ~98% and ~71–76% between the different isoforms, indicating specific roles for each isoform in vivo,[5] though distinct effects of vascular derived TGF-β1 and TGF-β3 have not been elucidated.

Following its cleavage from an inactive precursor, TGF-β forms a dimer that binds the TGFβ-receptor II on the endothelial surface. Subsequent recruitment of the TGFβ-receptor I (TGFβRI – ALK1 or ALK5 in ECs) into a tetrameric complex results in phosphorylation of the intracellular domain of TGFβRI, and the activation of smad transcription factors. Both TGF-β1 and TGF-β3 are detected in plasma in the absence of pathology, consistent with a role for these factors in vessel homeostasis,[6–8] as they play for epithelial cells.[9] In support of this notion, it has been shown that preeclampsia, which is characterized by systemic endothelial dysfunction, multiple end-organ ischemia, hypertension, proteinuria and increased vascular permeability, is due at least in part to elevated levels of soluble endoglin (sEng), which neutralizes TGF-β1 and TGF-β3.[10] Similarly, experimental systemic inhibition of TGF-β1 and TGF-β3, achieved by overexpression of sEng, leads to vascular permeability and perfusion defects as well as apoptosis of both vascular and non-vascular tissues.[11] sEng overexpressing mice also demonstrate an essential role for TGF-β in maintaining the endothelium in a non-activated state[12] and in maintaining microvessel integrity and function in the retina and choroid plexus.[13,14] Previous reports demonstrate that TGF-β signaling between EC and mural cells participates in vessel stabilization in vivo and in vitro, and paracrine TGF-β signaling between ECs and surrounding mural cells and astrocytes is well documented.[11,15–17]

Members of the KLF family of transcription factors, in particular KLF2, act as central mediators of shear stress induced changes in EC.[18–20] Shear stress-induced KLF2 regulates the expression of genes important in inflammation, thrombosis, and vessel tone, with an estimated ~46% of flow-regulated genes downstream of KLF2 induction.[21–25] Among the KLF2-inducible factors regulating vessel tone is eNOS, the well-described shear stress inducible enzyme that is responsible for formation of nitric oxide (NO). NO, a gas, with a half-life of a few seconds, acts upon underlying mural cells (pericytes and smooth muscle cells) to regulate vessel tone and on ECs to modulate inflammatory properties.[26–28] The precise mechanism by which the endothelium senses shear stress to increase both KLF2 and NO signaling is unclear.

Experimental inhibition of TGF-β by overexpression of sEng in vivo reveals an important role for TGF-β in maintaining vessel structure and function in the kidney, retina, mesentery and choroid plexus,[10,12–14] however the role of flow in modulating TGF-β signaling in ECs is poorly understood. We therefore investigated the effect of shear stress on TGF-β signaling in ECs and examined the relationship among flow-induced changes in TGF-β, KLF2 and NO signaling. Our results indicate that protective effect of shear stress on ECs is mediated via the TGF-β3 signaling and downstream KLF2 and NO signaling.

Methods

Detailed information on the materials and methods, including cell culture, RNA isolation and real-time–PCR, Western blot analysis and immunofluorescence are described in the Supplemental Material (available online at http://atvb.ahajournals.org).

Results

Role of TGF-β signaling in the morphological response of HUVECs to shear stress

Confluent monolayers of HUVECs were exposed to shear (10 dynes/cm^2) in serum-reduced medium and cell morphology was examined every 24 hr for three days. Consistent with previous observations,[29] shear stress caused HUVECs to align in the direction of the flow path, with the cell alignment becoming more pronounced with time (Figure 1). In contrast, static cells detached from the plate so that after three days only about ~50% of the cells remained (Figure 1).

To examine the role of TGF-β receptor-ALK5 signaling, HUVECs in a static and hemodynamic environment were exposed to 10 μM SB525334 in the presence or absence of shear stress. The effect of SB525334 on static HUVECs was variable, with either no effect or a slight attenuation of cell loss compared to static controls (Figure 1). Addition of SB525334 to cells prior to exposure to shear stress led to a modest loss of cells at 24 or 48 hr and a dramatic loss of cells after 72 hr (Figure 1 and Figure 2A). The exposure of cells to shear stress in the presence of SB525334 (Figure 1) resulted in ~50% loss of cells, though throughout the well, the degree of cell loss per field of view varied from 0–100%. The inhibition of ALK4, ALK5 and ALK7 with 10 μM SB431542 did not impair endothelial morphology (supplemental Figure I). These results reveal that ALK5 is required to maintain the endothelial monolayer exposed to shear stress.

Effect of TGF-β receptor inhibition on HUVEC proliferation and apoptosis under shear stress

Consistent with previous reports, exposure of HUVECs to shear stress reduced cell loss when compared to HUVECs under static conditions.[330,31] SB525334 blockade of ALK5 signaling in HUVEC exposed to shear stress led to an increase in cell loss (Figure 2A). As cell number may reflect changes in cell death and/or proliferation, HUVEC proliferation was determined using a cell tracer assay. Despite the increased cell number compared to static control, shear stress decreased HUVEC proliferation by approximately 25%. Addition of SB525334 further decreased HUVEC proliferation under both static and shear stress conditions by approximately 40% (Figure 2B and 2C).

Exposure of HUVEC to shear stress for 72 hr protected HUVEC from apoptosis and inhibition of TGF-β signaling prevented this protection (Figure 2D). Whereas addition of SB525334 had no significant effect on the level of apoptosis in cells grown under static conditions, it led to an approximately five-fold increase in apoptosis compared to shear stress control cells. Immunofluorescent localization of cleaved caspase-3, a key component of caspase-dependent apoptosis, revealed that shear stress decreased the cleavage of caspase-3 in HUVEC (Figure 2E). These findings demonstrate that ALK5 limits endothelial apoptosis under shear stress conditions.

Effect of shear stress and TGF-β receptor inhibition on TGF-β signaling in EC

In order to assess the effect of shear stress on TGF-β levels in HUVECs, conditioned media and cell lysates were collected after 72 hr and examined for active and total TGF-β1 and TGF-β3. Both latent and active TGF-β1 and TGF-β3 were detected in cell lysates, with active TGF-β3, but not TGF-β1, significantly increased by exposure to shear stress (Figure 3A). Examination of conditioned media revealed that neither total TGF-β1 nor total TGF-β3 were significantly increased under shear stress conditions; neither active TGF-β1 nor TGF-β3 were detected in media collected. Latent TGF-β1, but not TGF-β3 (latent or active), was detected in media and the levels were not changed with shear stress (Figure 3A). Real time PCR revealed that shear stress induced a nearly three-fold increase in TGF-β3; levels of TGF-β1 mRNA were unchanged. The induction of TGF-β3 by shear stress was blocked by the addition of SB525334 (Figure 3B). In contrast to increased TGF-β in EC exposed to shear stress, levels of ALK5 mRNA were unchanged (Supplemental Figure II).

To determine if the shear stress-induced increases in the levels of TGF-β3 resulted in downstream signaling, intracellular smad2 proteins were examined following 24 hr of shear stress. Shear stress led to a 2.5-fold increase in the phosphorylation of smad2, in the absence of any significant changes in total smad2 levels (Figure 3C). Addition of SB525334 reduced the phosphorylation of smad2 under both static and shear stress conditions without altering smad2 levels. These results reveal that shear stress of HUVEC increases TGF-β3 and activates downstream smad2 signaling.

Role of KLF2 and NO in shear stress-induced TGF-β signaling in HUVECs

KLF2 is a key transcriptional regulator of flow-induced changes in ECs.[20] To determine if TGF-β signaling plays a role in flow-induced KLF2 signaling, HUVECs were treated with SB525334 under static and shear stress conditions and examined for changes in KLF2 mRNA and protein after 24 hr. Shear stress increased KLF2 mRNA (Figure 4A) and protein (Figures 4B and C), and the addition of SB525334 resulted in a significant (approximately 50%) decrease in both KLF2 mRNA and protein. siRNA targeting of ALK5 also led to a significant (approximately 30%) reduction of KLF2 mRNA (Supplemental Figure III). Addition of 0.1 ng/ml TGF-β3 induced KLF2 mRNA, which was partially reversible with siRNA targeting of ALK5 (Supplemental Figure IV).

NO signaling is the classic mediator of the vasoactive effects of shear stress in ECs. Exposure of HUVECs to shear stress increased eNOS protein phosphorylation (Serine 1179) by approximately 2.5 fold after 24 hr (Figure 4D). To determine if TGF-β plays a role in the

shear stress-induced formation and release of NO from HUVECs, the effect of TGF-β receptor inhibition was examined. Addition of SB525334 decreased eNOS phosphorylation in the absence of any changes in total eNOS protein (Figure 4D). Cell supernatants were examined for the stable NO breakdown product (NOx) using the Griess reaction. Shear stress of HUVECs resulted in an approximately 2-fold increase in NOx release, which was significantly inhibited by the addition of SB525334 (Figure 4E).

To determine if the morphological changes and increased apoptosis observed with the inhibition of TGF-β receptor in shear stressed HUVEC was mediated by decreased NO, the ability of exogenous NO to rescue the cells from these effects was assessed. Indeed, the addition of SNP (1 μM) prior to the initiation of shear stress reversed the of TGF-β receptor inhibition on HUVEC morphology and apoptosis (Figures 5A and B). Taken together, these results reveal that shear-induced TGF-β3 activation of ALK5 is upstream of KLF2-NO signaling.

Interaction between KLF2 and TGF-β signaling under shear stress

In order to assess the interaction and signaling sequence between the TGF-β and KLF2 pathways, TGF-β1, TGF-β3 and KLF2 mRNA were knocked down using siRNA pools prior to exposure to shear stress for 24 hr. The specific siRNA pools decreased the levels of shear stress induced TGF-β1, TGF-β3, and KLF2 mRNAs, 5-fold, 3-fold and 6-fold, respectively (Figure 6A, 6B and 6C). Whereas reduction in the levels of TGF-β1 or KLF2 did not significantly alter TGF-β3 mRNA, knock down of TGF-β3 led to a 4-fold decrease in KLF2 mRNA (Figure 6C).

To determine the relative contribution of TGF-β1, TGF-β3 and KLF2 to the protective effects of shear stress on HUVECs, each of these proteins was individually knocked down with siRNA and the cells were then exposed to shear stress for 72 hr. Reduction of TGF-β3 in HUVEC exposed to shear stress led to a disruption of the monolayer that was similar to that observed with the pharmacologic inhibition of TGF-β receptor (compare Figure 6D and Figure 5A) whereas the knockdown of TGF-β1 had no effect. KLF2 knockdown also led to a notable disruption the HUVEC monolayer under shear stress, although not as dramatically as TGF-β3 knockdown. Addition of SNP (1 μM) completely reversed the effects of KLF2 knockdown, whereas the effect of TGF-β3 suppression was partially rescued with SNP. While SNP limited HUVEC loss resulting from TGF-β3 inhibition, shear stress-induced HUVEC alignment was still significantly impaired. The suppression of TGF-β1 levels had no effect in the presence or absence of SNP (Figure 6D). Taken together, these results reveal that TGF-β3, not TGF-β1, is upstream of shear stress-induced KLF2.

Discussion

Physiologic shear stress has been shown to be a important protective stimulus for the endothelium, limiting apoptosis and proliferation while maintaining endothelial quiescence, and in particular, suppressing the expression of pro-inflammatory proteins.[22,32] Our results are consistent with this function and expand this concept by demonstrating a critical role for TGF-β signaling in mediating the protective effects of physiologic shear stress. Shear stress of ECs induces TGF-β3 expression and signal activation via the transmembrane receptor

TGF-βRI (ALK5). Pharmacologic inhibition of ALK5 prior to exposure to shear stress for three days led to a dramatic disruption of the endothelial monolayer and increased cell death. Specific knockdown of TGF-β3, but not TGF-β1, resulted in similar cell loss over the same time course. In line with these findings, TGF-β3, but not TGF-β1, was significantly up regulated under shear stress. These findings demonstrate a novel role for shear stress-induced TGF-β3.

Cyclic stretch has been reported to upregulate ALK-1, but not ALK-5 or TGF-βRII, in EC co-cultured with smooth muscle cells, but not in EC in monoculture,[33] and other studies demonstrate that cyclic stretch induces autocrine TGF-β1 via activation of the ERK pathway.[34] Reports of bovine aortic ECs exposed to different types of shear stress revealed induction of TGF-β1 mRNA via K+ channel activation,[35] with laminar and pulsatile flow having greater effects than oscillatory flow.[36] These findings suggest that TGF-β1 plays a role in atheroprotection since oscillatory flow strongly correlates with atheroprone regions of the vascular tree. Consistent with our observations recent studies of mouse embryonic EC reveal shear stress induction of KLF2 is an ALK5 dependent process.[37]

Prior studies have reported the activation of TGF-β1 by contact of ECs with pericytes or astrocytes in vivo and induction of TGF-β1 in endothelial cells exposed to shear stress.[35,38,39] However, in our study of EC monocultures exposed to shear stress neither TGF-β1 nor ALK5 were regulated by shear stress. Rather, shear stress of HUVEC led to increased levels of TGF-β3 mRNA as well as both active and total protein. TGF-β3 protein was detected in association with cells but not in the conditioned media. Latent TGF-β1 but not active was detectable in the conditioned media, and was not changed in response to shear stress. These findings suggest autocrine/paracrine/juxtacrine roles for TGF-β1 and/or TGF-β3.

To determine if there was a relationship between TGF-β signaling and flow-induced KLF2 and NO, we specifically targeted each component using siRNA. Knock down of TGF-β3, but not TGF-β1, prevented the shear stress induction of KLF2. KLF transcription factors are well-documented mediators of the protective effects of shear stress, and these results indicate that the induction of TGF-β3 by shear stress is at least partially responsible for the activation of the KLF2 signaling cascade. TGF-β3 signaling did not mediate shear-induced KLF4 (supplemental Figure V). KLF2 has been shown to enhance the anti-inflammatory properties of ECs,[40] and we have previously described that inhibition of TGF-β in vivo using sEng overexpression leads to a pro-inflammatory response of mesenteric ECs, further supporting the notion that these pathways modulate anti-inflammatory properties of the endothelium in vivo.[12]

Previous reports of overexpression of KLF2 in HUVECs under static conditions demonstrated activation of inhibitory smad signaling, a decrease in smad2 phosphorylation and decreased TGF-β1 and TGF-β2, but not TGF-β3.[41] These results suggest a feedback mechanism that is consistent with our finding that TGF-β3, but not TGF-β1, induced expression of KLF2 under shear stress. Moreover, siRNA knockdown of KLF2 did not significantly alter the levels of TGF-β1 or TGF-β3, demonstrating that KLF2 is downstream of TGF-β signaling.

Despite their structural and biological similarities, it is clear that the TGF-β isoforms have distinct roles. For instance, TGF-β3, but not TGF-β1, protects keratinocytes against apoptosis in vitro and in vivo.[42] Gene transfer of TGF-β3, but not TGF-β1, inhibits constrictive remodeling and luminal loss after coronary angioplasty.[43] The molecular basis of these differences is unknown, though it may be due, at least in part, to the differences in binding to TGF-β modulating factors. For example, binding to cell surface and extracellular matrix heparan sulfate is isoform-specific and is thought to modulate their accessibility and activity.[44] TGF-β1, but not TGF-β3, binds heparan sulfate, potentiating its biological activity by limiting proteolytic degradation and inactivation by binding to α2-macroglobulin.[44] Similarly, TGF-β latency-associated protein (LAP), which is bound to TGF-β in its inactive form, interacts more effectively with TGF-β3 than with the two other isoforms.[45] Recent studies reveal differences in binding affinity between the TGF-β isoforms and the TGF-β receptors. TGF-βRII binds TGF-β1 and TGF-β3 with similar affinity due to a conserved amino acid sequence; however, ALK5 binds TGF-β3 with much higher affinity that TGF-β1. The differential kinetics of ternary complex assembly persists with binary complexes, with 5-fold greater affinity of TGF-β3/TGF-βRII for ALK5, than TGF-β1/TGF-βRII.[46]

Developmental studies of large arteries revealed TGF-β3 localization in smooth muscle progenitor cells, but not in endothelium[47] and TGF-β1, but not TGF-β3, mRNA in lung mesenchymal and endothelial cells.[48] Although the role of TGF-β3 in angiogenic processes has not been elucidate, reports suggest elevated TGF-β3 may promote angiogenesis during development by increasing Flk-1 and CD31 expression,[49] whereas other reports demonstrate TGF-β1, -β2 and -β3 cooperate to facilitate tube formation.[50]

In order to assess the mechanism of shear stress-induced TGF-β signaling, we examined the effects of ALK5 inhibition on the well-documented shear-induced protective EC signaling components, NO and KLF2.[51–53] Shear stress of ECs activates eNOS enzyme activity via phosphorylation at serine1179, leading to NO release, which acts on the underlying mural cells to regulate vessel tone.[53–55] In addition, NO has been shown to protect ECs from apoptosis.[3,56,57] Our data show that shear stress leads to increased phosphorylation of eNOS and NO release. These effects were blocked by the inhibition of ALK5 and rescued by the NO donor SNAP, demonstrating a clear role for the TGF-β signaling in mediating the shear stress induction of NO and suggesting that the protective effects of TGF-β3 are mediated largely by NO. Consistent with these findings, eNOS-knockout mice display hypertension, impaired microvessel function and leukocyte adhesion,[58–65] which also occur in sEng overexpressing mice (systemic TGF-β inhibition),[12–14] suggesting that the phenotype in mice in which TGF-β3 is impaired is at least partially a result of decreased nitric oxide.

siRNA knockdown studies revealed that TGF-β3, but not TGF-β1, was responsible for protecting ECs exposed to shear stress. Inhibition of ALK5 significantly blocked the induction of KLF2 mRNA and protein by shear stress, demonstrating that ALK5 is upstream of KLF2 in the signaling cascade. Inhibition of NO with L-NAME did not effect either TGF-β3 or KLF2 mRNA (data not shown), indicating that NO is downstream of both ALK5 and KLF2. Addition of exogenous NO fully reversed the effects of ALK5 inhibition; however, while NO prevented cell loss due to TGF-β3 knockdown, it did not prevent the loss of EC alignment. These results suggest that the action of TGF-β3 to align EC along the

flow path is independent of NO signaling. Similarly, knockdown of KLF2 did not result in the same degree of cell loss as knockdown of TGF-β3 under shear stress, suggesting that KLF2 is not the only target for TGF-β3 signaling in ECs exposed to flow.

Further investigation of putative NO signaling activators induced by shear/TGF-β3 did not indicate a role for any of the previously described signaling molecules (pp-PLC-γ, PLC-γ, HSP90α, PI3k, pp-Akt, Akt, pp-ERK, ERK, pp-p38, p38, JNK, Mek5). Non-biased proteomic analysis, however, did reveal a number of novel proteins regulated by shear stress induction of TGF-β3, but not TGF-β1. For example, the level of RhoA was decreased in sheared ECs, whereas siTGF-β3 treated sheared cells exhibited a significant increase in RhoA. Interestingly, RhoA has been previously shown to inhibit NO signaling via multiple pathways.[66] ALK1 signaling in ECs stimulates migration and proliferation, both of which are inhibited upon ALK5/ALK1 complex formation. Proteomic analysis revealed the differential expression of two proteins that limit ALK1 signaling; shear stress increased EC PPP1CA (ALK1 inhibitor) and decreased CSNK2B (ALK1 enhancer), effects that were mediated by the activation of TGF-β3, but not TGF-β1.

We have described a novel effect of shear stress on EC in which the induction of TGF-β3 expression leads to activation of KLF2 and NO signaling. Understanding the mechanisms underlying this effect sheds light on the potential homeostatic role for plasma TGF-β3 and should enable targeting of the TGF-β pathway in pathologies in which TGF-β3 signaling is perturbed such as preeclampsia and cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non standard abbreviations & and acronyms

ECs	Endothelial cells
HUVECs	Human umbilical vein endothelial cells
KLF	Krüppel-like factor
eNOS	endothelial nitric oxide synthase
SNAP	S-nitroso-N-acetylpenicillamine
sEng	soluble endoglin
NO	nitric oxide

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Significance

Vascular endothelial cells (ECs) are continuously exposed to blood flow that contributes to the maintenance of vessel structure and function; however, the effect of hemodynamic forces on TGF-β signaling in the endothelium is not well understood. We have described a novel effect of shear stress on EC, in which the induction of TGF-β3 expression leads to activation of KLF2 and NO signaling, which are known to limit endothelial dysfunction and maintain endothelial homeostasis. The induction of TGF-β by shear stress was specific to TGF-β3 - did not effect TGF-β1, thus revealing their differential regulation. Each of the TGF-β's is strongly conserved across species, however their distinct roles are poorly described. Understanding the mechanisms underlying these changes sheds light on the potential homeostatic role of plasma TGF-β3 and should enable targeting the TGF-β pathway in pathologies in which TGF-β3 signaling is perturbed such as preeclampsia and cancer

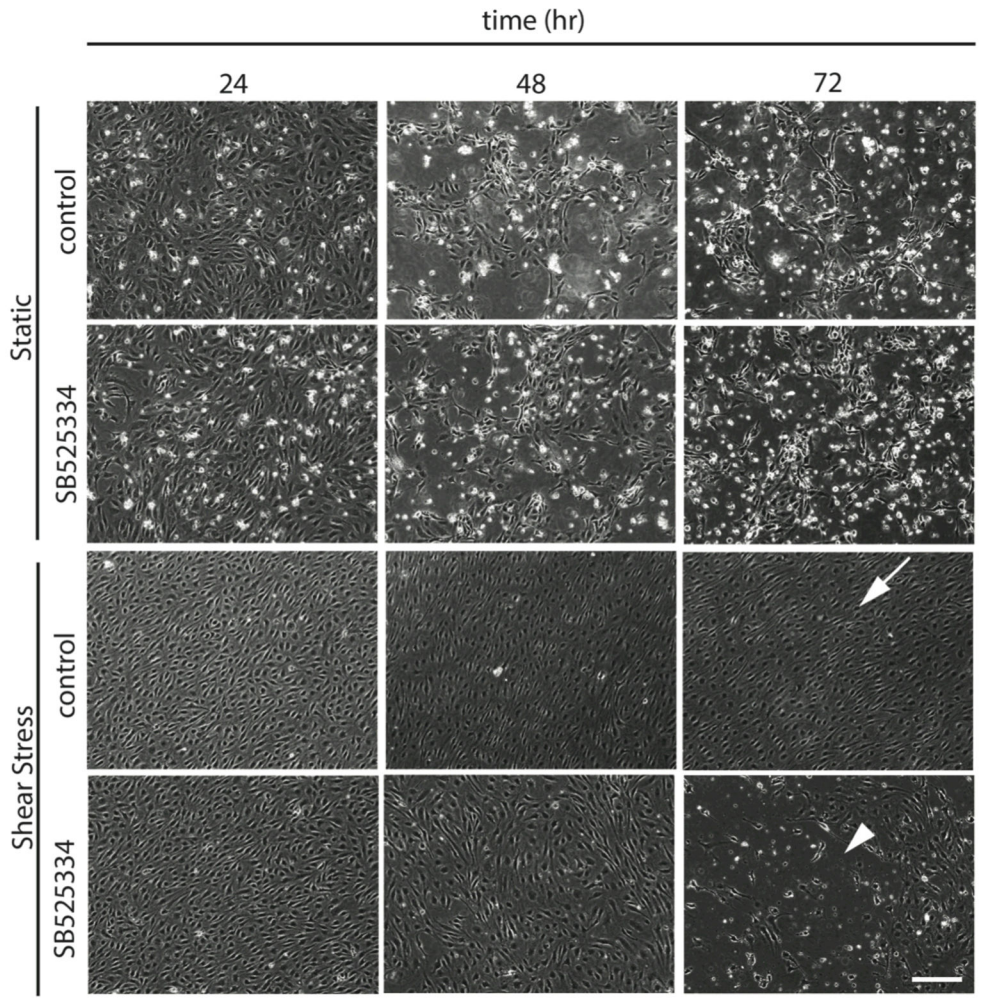

Figure 1.

Effects of ALK5 inhibition on shear stress-induced changes in HUVEC morphology. Shear stress led to HUVEC alignment along the flow path (arrow). Treatment of cells with the ALK5 inhibitor, SB525334, led to a disruption of the HUVEC monolayer after 72 hr (arrowhead).

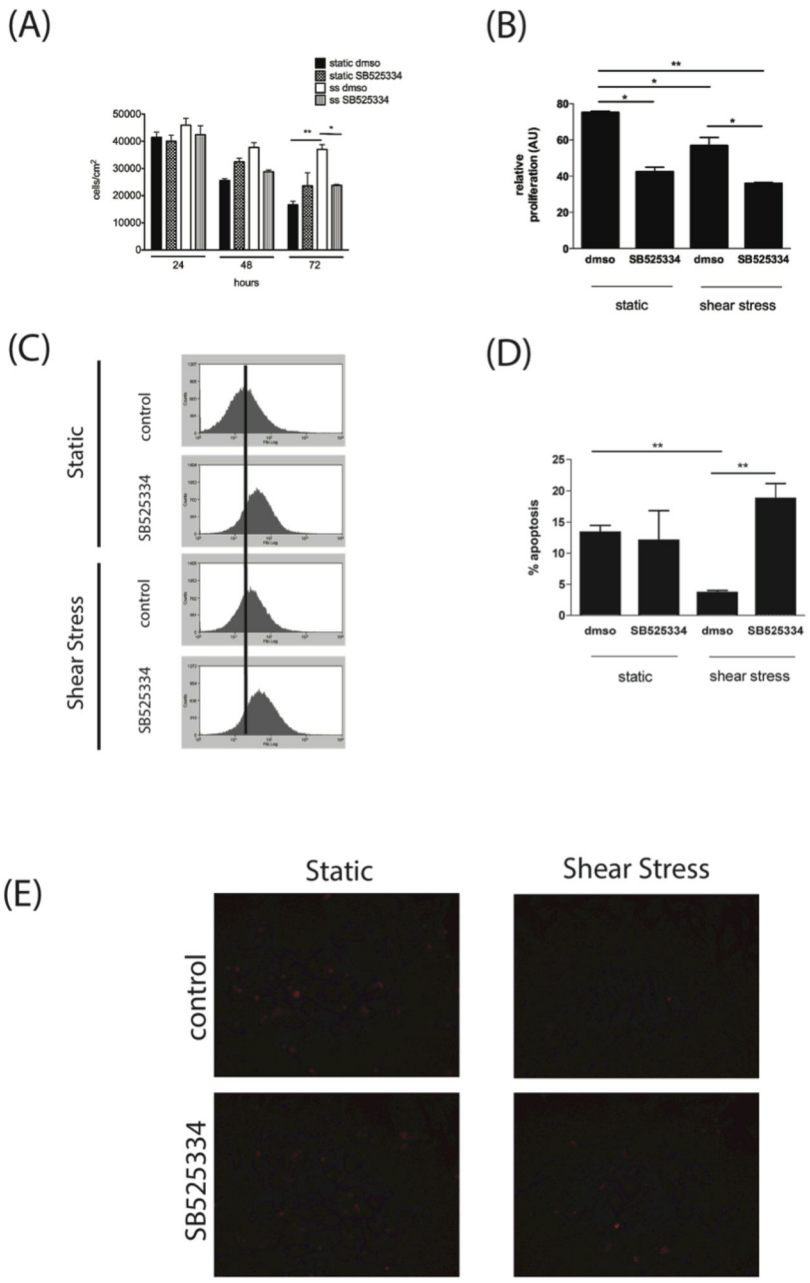

Figure 2.

(A) Shear stress of HUVEC limited the decrease in cell numbers compared to cells grown under static conditions; SB525334 reduced this protective effect. (B)(C) Shear stress decreased HUVEC proliferation; SB525334 further decreased proliferation. (D) Shear stress decreased HUVEC apoptosis and caspase 3 activation (E) and is dependent on ALK-5.

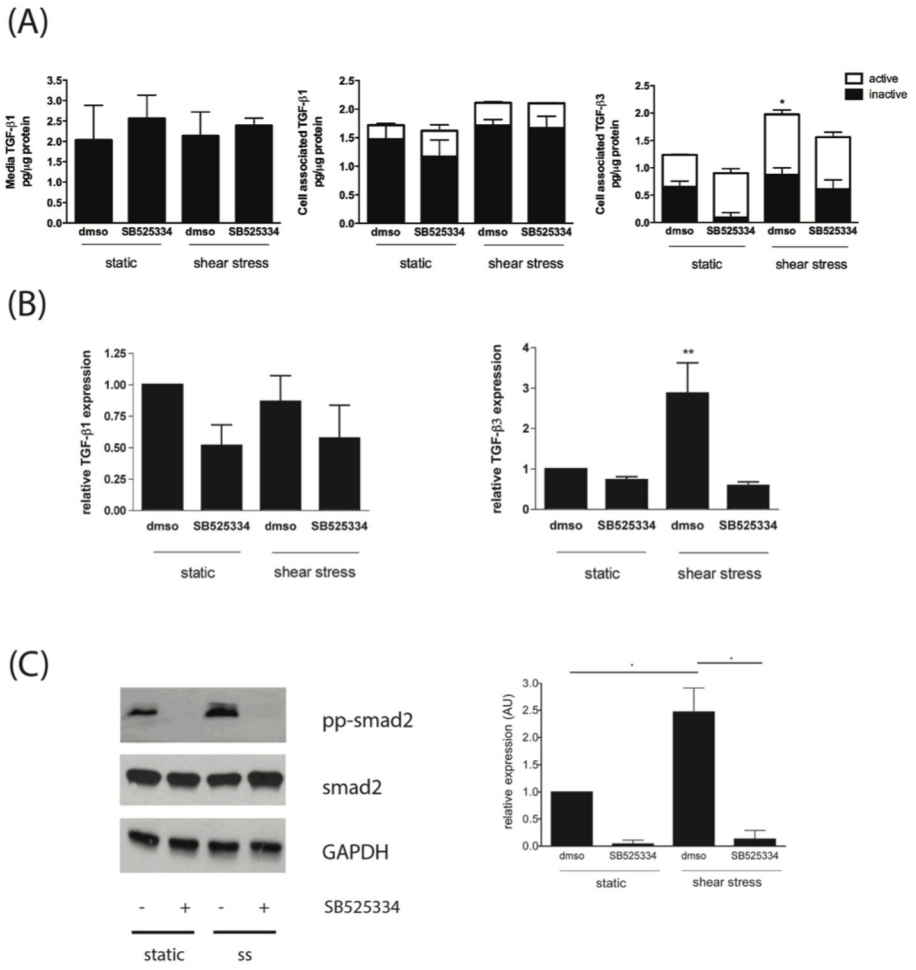

Figure 3.

(A) TGF-β1 was detected in HUVEC conditioned media. Shear stress increased active and latent cell associated TGF-β3, but had no effect on levels of TGF-β1. (B) Shear stress increased TGF-β3 mRNA and phosphorylation of smad2 (C). Addition of SB525334 inhibited the phosphorylation of smad2.

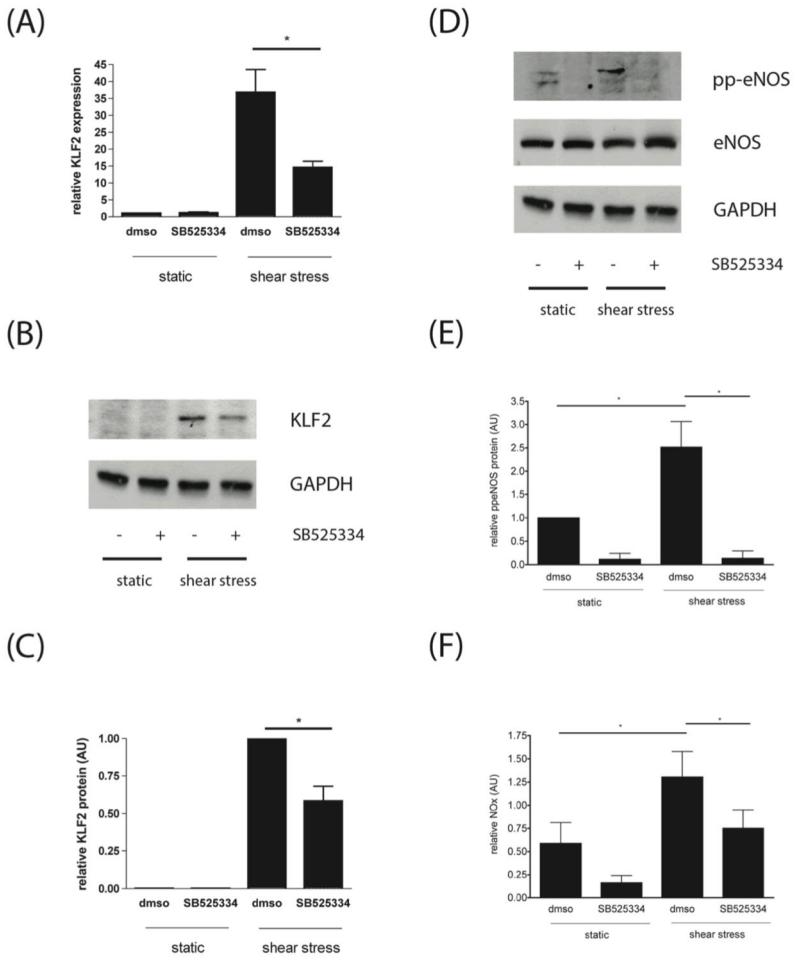

Figure 4.

Shear stress increases HUVEC KLF2 mRNA (A) and protein (B) (C); this effect was partially reversed by the inhibition of ALK5. Shear stress increased phosphorylation of eNOS protein (D) (E) and release of NOx (F), which was blocked with inhibition of ALK5.

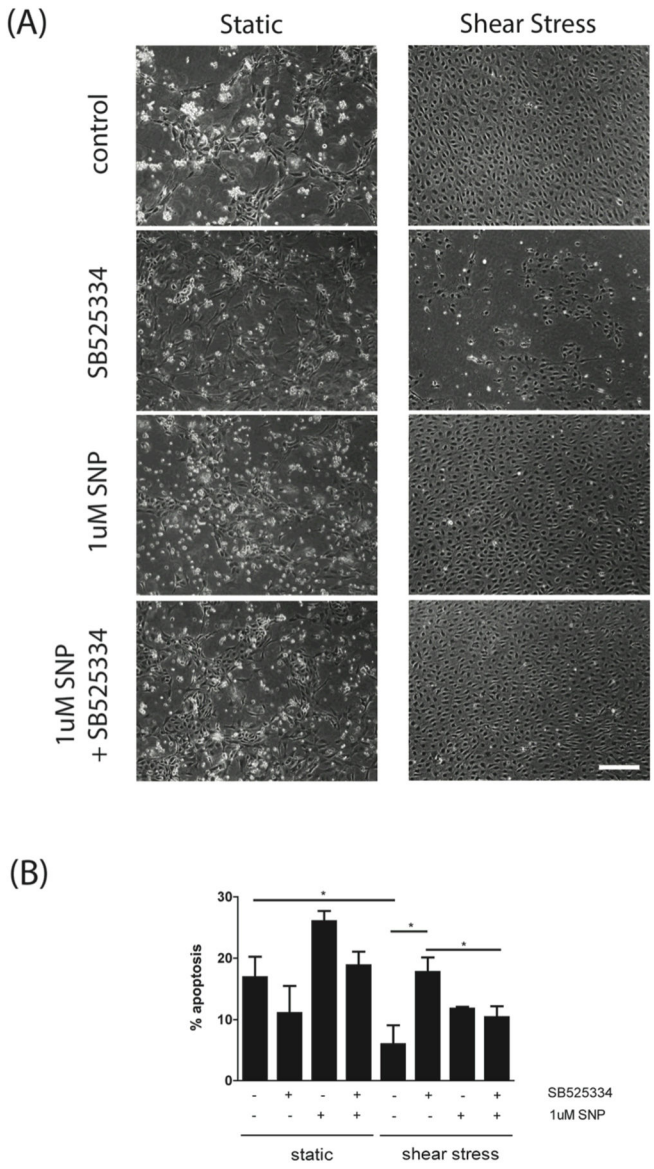

Figure 5.

(A) SB525334 prevented the protective effects of shear stress on HUVECs at 72 hr and addition of 1 uM SNAP reversed this effect. (B) FACs analysis of apoptotic HUVECs revealed that the addition of SNAP prevented the increase in HUVEC apoptosis caused by the blockade of ALK5.

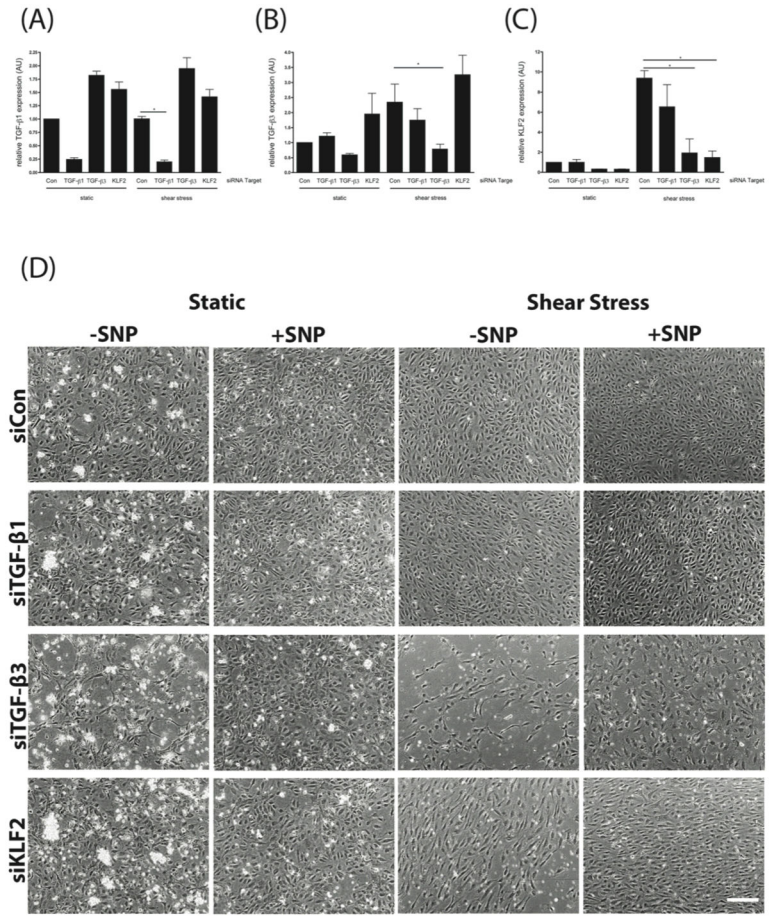

Figure 6.

mRNA levels of (A) TGF-β1, (B) TGF-β3, and (C) KLF2 following siRNA treatment. (D) siTGF-β3 significantly disrupted the HUVEC monolayer. SNP rescued this cell loss, but not loss of cell alignment. siKLF-2 partially impaired HUVECs morphology, which was fully rescued by the addition of exogenous SNP.