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Effects of Biomechanical Stress on Gene Regulation in Vascular Cells

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ABSTRACT

The vascular vessel wall is constantly exposed to biomechanical forces, such as shear and tensile stress. Biomechanical forces are important for several physiological and pathological processes and have been shown to regulate a number of fundamental vascular functions, such as vascular tone and remodeling processes. The aim of the present thesis was to study the effects of biomechanical forces on the vessel wall.

Intact human conduit vessels were exposed to normal or high intraluminal pressure, or low or high shear stress in combination with a physiological level of the other factor in a unique vascular *ex vivo* perfusion model, developed in our laboratory. Global gene expression profiling was performed with microarray technology of endothelial cells from stimulated vessels. Biomechanical forces were found to regulate a large number of genes. The fraction of genes that responded to both pressure and shear stimulation was surprisingly low, which indicates that the two different stimuli induce distinct gene expression response patterns. Further, these results suggest that the endothelium has the capacity to discriminate between shear stress and pressure stimulation.

Detection and quantification of changes in gene expression require valid and reliable endogenous references genes. Therefore, the appropriateness of ten reference genes for studies of biomechanically stimulated endothelium was evaluated by microarray technology and real-time RT-PCR.

Shear stress plays an essential role in regulation of vascular tone and remodeling, and P2 receptors have been suggested to be mediators of some of these effects. We therefore studied the effects of shear stress on P2 receptor expression in intact human vessels. In the endothelium, no significant regulation of P2 receptor mRNA levels was observed. However, in smooth muscle cells, high shear stress decreased mRNA expression of the contractile $P2X_1$ receptor and increased the mitogenic $P2Y_2$ and $P2Y_6$ receptors. These findings were consistent at the protein level with Western blot analysis and morphologically with immunohistochemistry. This suggests that the shear force can be transmitted to the underlying smooth muscle cells.

The interplay of shear stress and inflammatory stress on urokinase-type plasminogen activator (u-PA) and plasminogen activator inhibitor-1 (PAI-1) expression was studied in an *in vitro* shear stress system. Endothelial cells were exposed to either shear stress, the proinflammatory cytokine tumor necrosis factor-α (TNF-α), or a combination of both. High shear stress markedly reduced u-PA expression whereas TNF-α induced u-PA expression. Combining shear stress and inflammatory stimulation reduced the TNF-α mediated u-PA induction, which suggests that shear stress exerts a strong protective effect. The TNF-α induced expression was proposed to be partly mediated by activation of c-jun N-terminal kinase (JNK). The PAI-1 expression was induced both by shear stress and TNF-α, and the effect was potentiated when the two stimuli were combined.

In conclusion, these findings illustrate that biomechanical forces regulate a large number of genes in the endothelium and that shear stress and pressure induce distinct expression patterns. Shear stress also has the capacity to influence gene expression in smooth muscle cells in intact vessels and protect against inflammatory stress, which illustrates its potency as a regulator of endothelial cell function.

Key words: shear stress, intraluminal pressure, endothelium, gene expression, DNA microarray, real-time RT-PCR , reference genes, smooth muscle cells, P2 receptors, TNF-α, urokinase-type plasminogen activator

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LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, identified in the text by their Roman numerals:

I **Andersson M**, Karlsson L, Svensson P-A, Ulfhammer E, Ekman M, Jernås M, Carlsson L, Jern S. Differential Global Gene Expression Response Patterns of Human Endothelium Exposed to Shear Stress and Intraluminal Pressure.
Journal of Vascular Research 2005;42:441-52

II Dourodi R*, **Andersson M***, Svensson P-A, Ekman M, Jern S, Karlsson L. Methodological Studies of Multiple Reference Genes as Endogenous Controls in Vascular Gene Expression Studies.
* Both authors contributed equally
Endothelium 2005;12:215-23

III Wang L, **Andersson M**, Karlsson L, Watson M-A, Cousens D, Jern S, Erlinge D. Increased Mitogenic and Decreased Contractile P2 Receptors in Smooth Muscle Cells by Shear Stress in Human Vessels with Intact Endothelium.
Arteriosclerosis, Thrombosis, and Vascular Biology 2003;23:1370-76

IV **Carlström M**, Ulfhammer E, Larsson P, Bergh N, Jern S, Karlsson L. Protective Effect of Laminar Shear Stress on u-PA Expression in Vascular Endothelial Cells Exposed to Inflammatory Stress.
In manuscript

CONTENTS

ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
AP-1	activator protein-1
ATF-2	activating transcription factor-2
ATP	adenosine triphosphate
bp	base pair
cDNA	complementary DNA
CT	threshold cycle
CYC	cyclophilin
EDHF	endothelium-derived hyperpolarizing factor
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
eNOS	endothelial nitric oxide synthase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HAEC	human aortic endothelial cells
HPRT	hypoxanthine-guanine phosphoribosyl transferase
HUVEC	human umbilical vein endothelial cells
IL-1β	interleukin-1β
JNK	c-jun N-terminal kinase
MMP	matrix metalloproteinase
MAPK	mitogen activated protein kinase
MAS 5	Microarray Suite 5
mRNA	messenger RNA
NF-κB	nuclear factor-κB
NO	nitric oxide
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate buffered saline
PEA3	polyoma enhancer site A3
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SMC	smooth muscle cell
TBS	tris buffered saline
TfR	transferrin receptor
TIMP	tissue inhibitor of metalloproteinase
TNF-α	tumor necrosis factor-α
t-PA	tissue-type plasminogen activator
UDP	uridine diphosphate
u-PA	urokinase-type plasminogen activator
u-PAR	urokinase-type plasminogen activator receptor
UTP	uridine triphosphate
VCAM-1	vascular cell adhesion molecule-1
vWF	vonWillebrand factor

INTRODUCTION

Cardiovascular disease is the leading cause of death in the Western world. Acute events, such as myocardial infarction and ischemic stroke are usually triggered by rupture of an atherosclerotic lesion and subsequent formation of a flow-arresting thrombus. A number of risk factors for atherosclerosis have been identified, including hypercholesterolemia, smoking, and hypertension. Further, the associated inflammatory reaction has been closely implicated both in the development of atherosclerosis *per se*, and in the complicating atherothrombotic event. However, despite the fact that these factors mainly act on a generalized systemic level, atherosclerotic lesions typically show a distinct and highly diversified pattern of anatomic localization and are usually confined to specific areas in the blood vessel.

Interestingly, this heterogeneity of lesion distribution has been found to be closely correlated with the specific hemodynamic profile acting on each region of the vessel. In addition, biomechanical forces have also been shown to regulate a number of physiological functions in the vessel wall, such as vascular tone by release of vasoactive substances, vascular adaptation and remodeling by growth factors, formation of new blood vessels, and the vessel's thromboprotective mechanisms. Hemodynamic factors have also been suggested to be involved in the pathophysiological processes that may lead to plaque rupture and atherothrombosis through their effects on hemostasis and proteolysis. Taken together, these observations indicate that biomechanical stress is of pivotal importance both in vascular physiology and pathology.

The vascular vessel wall

The wall of arteries and veins consists of three layers; tunica intima, tunica media, and tunica adventitia. The tunica intima consists of a monolayer of endothelial cells lining the lumen of the vessel and is supported by a subendothelial layer of loose connective tissue. The tunica media is mainly composed of smooth muscle cells and extracellular matrix proteins. The tunica adventitia is composed of fibroblasts and loose connective tissue. The tunica media is thick in arteries and thin in veins (Figure 1). The surface area of the endothelium in humans has been reported to vary between 350 and 1,000 m^2 and with a weight of 0.1-1.5 kg [1-3]. The endothelium is actively involved in many functions such as control of vascular tone, fluid and solute exchange, hemostasis and coagulation, and inflammatory responses [1, 4, 5]. The endothelium also expresses fibrinolytic activity by synthesis of tissue-type plasminogen activator (t-PA) [6, 7] and urokinase-type plasminogen activator (u-PA) [8]. The primary function of the vascular smooth muscle cells is contraction and relaxation and thereby regulation of vessel diameter, but through their mitogenic responses they are also involved in remodeling of the blood vessel in response to adaptive stimuli.

Biomechanical forces

The vessel wall is constantly exposed to biomechanical forces. There are two major types of biomechanical forces acting on blood vessels; shear stress and tensile stress

(Figure 1). Wall shear stress is the frictional force on the endothelium exerted by the blood flow. Shear stress is determined by blood flow, vessel geometry, and fluid viscosity, and is expressed in force unit per unit area (dynes/cm^2) [9]. Shear stress varies in the vascular tree, ranging between 1 - 6 dyn/cm^2 in veins and 5 - 40 dyn/cm^2 in large arteries [9-12]. Typically, shear stress is maintained at a level of 10 - 15 dyn/cm^2 in normal conduit arteries [13, 14]. Tensile stress is created by blood pressure and acts circumferentially on the vessel wall [10, 14]. Tensile stress, or strain in large arteries ranges from 2 to 18% during the normal cardiac cycle [15]. The hydrostatic pressure imposes a compressive stress on the vessel wall [16], but due to the counter-acting tissue pressure, compression does not cause a deformation of the cells.

Biomechanical stress modulates intracellular signaling, and gene and protein expression, which results in a functional control of vascular tone and vessel wall structure. Vascular tone is regulated by shear-induced release of vasodilators and vasoconstrictors, such as nitric oxide (NO), prostacyclin and endothelin-1 [17, 18]. Changes in vessel wall structure is associated with a redistribution of the extracellular matrix and smooth muscle cells in the media [19-21]. Furthermore, local hemodynamic forces play a major role in the regional localization of atherosclerosis [22]. Atherosclerotic lesions are often present at locations close to vascular branch points and bifurcations [23], where the flow rate is low and the flow pattern disturbed [24, 25]. The first evidence that low shear stress was involved in localization of atherosclerosis was described 1969 by Caro *et al*. [26]. Further, experiments in animal models also support the atherogenic role of low shear stress [22, 27, 28].

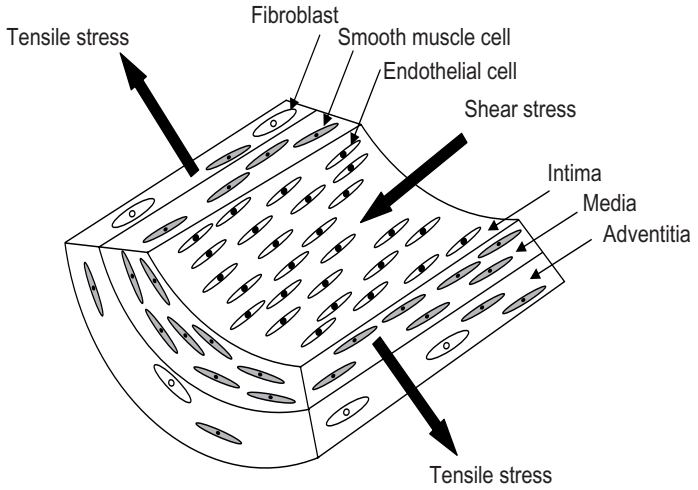


Figure 1. Schematic representing the major types of biomechanical forces acting on the blood vessel wall. Shear stress is the frictional force exerted by the blood flow. Tensile stress is generated by the blood pressure. The vessel wall consists of three layers with endothelial cells, smooth muscle cells and fibroblasts.

Flow is mainly considered to affect the endothelium, but vascular smooth muscle cells are also suggested to indirectly or directly be subjected to shear stress, which result in regulation of gene expression and migration [12, 29]. In the presence of an intact endothelium, the level of shear stress acting on the smooth muscle cells has traditionally been thought to be too low to influence its function. However, studies in fluid dynamic models indicate that smooth muscle cells (SMCs) may be exposed to levels of shear stress that are high enough to modulate their gene expression [29, 30]. In these models the direct effect of shear stress is suggested to be a result of the interstitial flow through fenestral pores in the internal elastic lamina [29, 30]. *In vitro* studies have shown that SMCs are responsive to shear stress in the range of 1 - 25 dyn/cm^2 and regulate the synthesis of transforming growth factor β, t-PA [31], heme oxygenase-1 [32], NO [33], and prostaglandins [34].

Endothelial and smooth muscle cells are equipped with numerous receptors that allow them to detect and respond to the mechanical forces generated by pressure and blood flow [10, 35]. Mechanoreceptors at the luminal surface of the endothelium include ion channels, integrins, G-protein linked receptors, and tyrosine kinase receptors [10, 11, 36]. In particular, integrins and membrane K^+ channels have been proposed to function as endothelial mechanotransducers [11, 37, 38]. Activation of mechanoreceptors releases second messengers such as focal adhesion kinase, phosopholipase C, inositol 1,4,5-triphosphate (IP_3), and mitogen-activated protein kinase (MAPK) cascades, that are able to activate MAP kinases [39, 40]. Extra-cellular signal-regulated kinase (ERK) 1/2 and c-jun N-terminal kinase (JNK) MAP kinase pathways have been reported to be activated, which down-stream leads to transcription factor activation and translocation to the nucleus [41-43]. Another signaling mechanism involves direct force transmission through the cytoskeleton, from the cell membrane to the nucleus, intracellular junctions, and focal adhesion points [44].

Vascular tone and remodeling

Biomechanical forces are important regulators of vascular tone by mediating release of vasoactive substances such as NO and endothelin-1 [45] and induce adaptive processes of the vessel wall [21]. Lately, P2 receptors have also been reported to play an important role in both regulation of flow control and vascular remodeling *e.g.* by smooth muscle cell proliferation [46-48]. P2 receptors mediate the action of extracellular nucleotides, ATP, ADP, UTP, and UDP, and are distributed throughout the entire body, including the vessel wall. The P2 receptors are divided into two classes, based on their signal transduction mechanisms and their characteristic molecular structure [49]. The P2X receptors are ligand-gated intrinsic ion channels and the P2Y receptors are G-protein coupled receptors. So far, the P2 family comprises seven P2X subtypes ($P2X_1$-$P2X_7$) [50] and eight P2Y subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$), that have been cloned, characterized, and recognized to respond to stimulation by extracellular nucleotides in humans [36, 51].

Endothelial cells are regulated by nucleotides released from platelets, endothelial cells, neurons, and damaged cells [48]. Shear stress, hypoxia, and inflammation are important stimuli of ATP and UTP release from endothelial cells [52-56]. Vascular tone is

regulated by P2 receptors, both by vasodilation when ATP in the vascular lumen acts on endothelial cells to release NO [57], endothelium-derived hyperpolarizing factor (EDHF) [58, 59] and prostacyclin [60, 61], and also by contraction of SMCs when ATP is released from sympathetic neurons on the adventitial side or when released in the lumen when the endothelium is damaged [62].

It has been suggested that mainly the activation of P2Y receptors induce endothelium-dependent vasodilatation [63, 64]. Among the P2Y receptors, $P2Y_1$, $P2Y_2$, and $P2Y_6$ seem to be of major importance [64-66]. However, recently, $P2X_4$,which is by far the most abundantly expressed P2X receptor in the endothelium [66-68], was reported to be involved in regulation of vascular tone [69]. $P2X_4$-deficient mice were shown to have an impaired NO production and elevated blood pressure compared to wild-type mice [69].

ATP is released together with noradrenaline from sympathetic neurons and acts on vascular smooth muscle cells to cause vasoconstriction [48, 65]. $P2X_1$ is the main vasoconstrictor among the P2X receptors [70] and also the most abundantly expressed [66]. However, a contractile effect of UTP mediated by $P2Y_2$ and UDP by $P2Y_6$ has been observed [71] . Contraction of SMCs is also mediated by ADP binding to $P2Y_{12}$ receptors [72]. ATP is rapidly degraded into ADP, AMP, and adenosine by ectonucleotidases to terminate signaling and reduce the contractile effect [73, 74]. Extracellular ATP, UTP, and UDP are potent growth factors for vascular SMCs by activation of P2Y receptors [75, 76], which results in stimulation of DNA synthesis, protein synthesis, increased cell number, immediate-early gene expression, cell-cycle proliferation, and tyrosine phosphorylation [77, 78]. The trophic effect on vascular SMCs implicate that extracellular ATP signaling may play a role in the development of atherosclerosis, and possibly also hypertension [77].

Hemostasis and proteolysis

Biomechanical forces also play an important role in regulating fibrinolysis, coagulation, and proteolysis [79-82]. The function of the fibrinolytic system is to degrade fibrin, and thus dissolve thrombotic material. The two major plasminogen activators, u-PA and t-PA, catalyze the conversion of plasminogen into plasmin, which in turn degrades fibrin [83]. t-PA is the key enzyme in the intravascular fibrinolysis, while u-PA mainly functions within tissues, which results in tissue remodeling and cell migration [84]. u-PA has been implicated in many biological functions, such as wound healing, tumor metastasis, inflammation and lately atherosclerosis [85, 86]. u-PA is a serine protease that binds to a specific cellular receptor, the urokinase-type plasminogen activator receptor (u-PAR), which results in enhanced activation of cell-bound plasminogen [84, 87]. The main inhibitor, plasminogen activator inhibitor 1 (PAI-1) inactivates u-PA [83].

The zymogen single-chain u-PA is secreted from the cells and forms a complex with cell membrane bound u-PAR. Single-chain u-PA is then cleaved by plasmin and kallikrein to the active two-chain u-PA [83]. When u-PA is degraded, the proteolytic activity of u-PAR bound u-PA is blocked by binding to PAI-1, subsequently, the

u-PA-u-PAR-PAI-1 complex is internalized and u-PA is degraded, while the u-PAR receptor is recycled to the cell surface [88-90]. Thus, PAI-1 controls cell-associated u-PA by suppressing its proteolytic activity and by reducing the amount of surface-bound u-PA.

The u-PA gene minimal promoter contains a TATA box and several GC boxes. Transcription of the human u-PA gene is modulated by a inducible enhancer located at -2,0 kb [91]. The enhancer contains an upstream PEA3/AP-1a site and a down-stream AP-1b site [92]. AP-1 elements recognize the transcription factor AP-1, which is either a homodimer of Jun or a heterodimer of Jun and Fos [24]. Transcription factors binding to the PEA3/AP-1a and AP-1b sites are activated by members of the MAP family [93].

u-PA is synthesized by many cell types, including smooth muscle cells, macrophages, and endothelial cells [8, 94, 95] and can be induced by different stimuli, such as growth factors and cytokines [96, 97]. Expression of u-PA has been shown to be increased in atherosclerotic human aortas, carotid arteries, and coronary arteries [85, 94, 98]. u-PA can stimulate the migration and proliferation of smooth muscle cells [99] and mediates arterial neointima formation [100]. Overexpression of u-PA in the arterial wall was shown to cause acute vascular constriction and accelerated atherosclerotic lesion growth [101]. Apart from this, u-PA also seems to have the potential to degrade elastin and in this way possibly make the arterial wall more prone to aneurysm formation [102]. Further, u-PA mediated plasmin activates several matrix metalloproteinases that in turn cause extracellular matrix degradation [103].

The endothelial expression of u-PA is low, but can be induced by inflammation [104]. Therefore, the impact of endothelial u-PA expression is probably enhanced during inflammation. Since hemodynamic factors, together with inflammation, are important for development of atherosclerosis [105], studies of the combined effect of these two factors may provide valuable information on the process. However, the interplay between shear stress and inflammation in modulating endothelial gene expression and function has not been fully clarified. Proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), mediate systemic inflammation and induce the inflammatory response of the endothelium by enhancing adhesion molecules and secretion of inflammatory mediators [106]. Further, TNF-α stimulation of the endothelium has been shown to induce u-PA expression [107], while u-PA expression has been reported to be reduced by shear stress [82]. However, the knowledge of the combined stimulation is incomplete.

Methodological challenges in studies of biomechanical forces

Cellular responses to shear stress and tensile stress have traditionally mainly been studied with cultured cells in different types of *in vitro* systems. For studies of shear stress, parallel-plate flow chamber [108, 109] and cone-and-plate device systems [110, 111] are some of the most widely used experimental systems. Tensile stress is usually studied by using a device in which the cells are exposed to cyclic stretch [112]. The advantages of *in vitro* systems are that the biomechanical stress can be exactly defined

and that the use of molecular biological techniques is more easily applied to cultured cells due to the abundant amount of material that can be obtained. On the other hand, the potentially important cross-talk between endothelial cells and smooth muscle cells can not be studied and there is always a risk that cells may loose or gain functions when cultured *in vitro*. *In vivo* experiments are typically based on perfusion studies of isolated organs, for instance the human forearm [113, 114]. The pressure and flow conditions are physiologically relevant, but in such a system it is impossible to define the exact force each vessel is exposed to. This is particular true for shear stress, since shearing forces are extremely difficult to measure *in vivo*. In addition, gene expression studies of human endothelium *in vivo* are impossible to perform. To overcome some of the limitations of these traditional approaches, an *ex vivo* vascular perfusion system has been developed in our laboratory, in which intact human vessels can be perfused under controlled biomechanical conditions to simultaneously investigate the distinct effects of tensile and shear stress [115, 116].

In addition to the problems in selecting an adequate and close-to-physiology stimulation experimental set-up, evaluation of the effector variables also poses some challenges. In particular, gene expression studies of biomechanically stimulated vascular cells require sensitive and reliable methods. Currently, real-time reverse transcription polymerase chain reaction (RT-PCR) is the most frequently used method for quantification of gene-specific mRNA. The method is accurate, fast, and allows quantification of low copy number mRNAs. Other methods for quantification of mRNA include Northern blotting, which is a semiquantitative method with low sensitivity that require large amount of RNA, and ribonuclease protection assay, which has a high specificity but a low capacity in quantification [117]. Before introduction of real-time RT-PCR, conventional RT-PCR was used for gene-specific mRNA quantification [118]. However, the use of end-point PCR product analysis makes it difficult to accurately determine the initial quantity of template molecules, since the amount of amplicon at the end of the amplification cycles depends not only on the input amount, but also technical variations during the PCR reaction. By contrast, real-time RT-PCR measure the amount of amplified PCR product during the exponential logarithmic phase, in which ideally there is a doubling of PCR product in every cycle [119, 120]. The input amount of gene-specific mRNA can then be calculated by either the standard curve method [121, 122] or the delta delta threshold cycle method ($\Delta\Delta C_T$ method) [123].

Normalization of gene-specific mRNA data is usually performed with an internal control [124]. Using endogenously expressed reference genes (frequently called housekeeping genes) as such controls require that they are constitutively expressed and that experimental conditions do not affect their expression [125]. Any variation in the reference gene will obscure real changes and thereby produce artifactual changes. Consequently, the reference gene needs to be validated for each experimental condition. The need for an appropriate and thoroughly validated reference gene is greater when quantification is based on highly sensitive methods such as real-time RT-PCR, compared to methods with low sensitivity such as Northern blotting.

The microarray technology is the most powerful method for gene expression studies, since it allows simultaneous analysis of tens of thousands of genes. Most studies on

the effect of biomechanical forces on gene expression have been performed on a single-gene basis. Furthermore, the microarray technique enables comprehensive screening of entire gene expression profiles. The types of microarray platforms mainly include cDNA microarray and oligonucleotide microarray [126, 127]. One of the most widely used commercial available microarray platform is the Affymetrix GeneChip. This technology uses multiple probe sets of each transcript to measure the amount of mRNA present [128]. In the present work, the Affymetrix microarray technology was used to compare the global gene expression patterns induced by shear stress and intraluminal pressure in the endothelium.

AIMS

Against this background, the overall objective of this thesis was to study the effects of biomechanical forces on gene expression in vascular cells. The specific aims were:

- to compare global gene expression responses induced by shear stress and intraluminal pressure stimulation of the endothelium in an intact human vessel (Paper I)

- to explore the hypothesis that shear stress and intraluminal pressure induce differential response patterns (Paper I)

- to evaluate appropriate reference genes for gene expression analysis of biomechanically exposed endothelium (Paper II)

- to study the effect of shear stress on P2 receptor expression in endothelial and smooth muscle cells (Paper III)

- to study the interplay of shear stress and inflammation on u-PA and PAI-1 expression in endothelial cells (Paper IV)

MATERIAL AND METHODS

Overview of experimental designs

Study I

Study I was designed to investigate the global gene expression responses of endothelium exposed to shear stress and intraluminal pressure. The *ex vivo* vascular perfusion system was used and intact human umbilical veins were exposed to high or low shear stress under normal pressure, or high or normal pressure under normal shear stress. Gene expression profiling was performed by Affymetrix microarray technology.

Study II

This study was designed to examine the expression of ten different reference genes in biomechanically stimulated vascular endothelium. Shear stress and intraluminal pressure experiments were performed in the *ex vivo* vascular perfusion system. Gene expression was analyzed by microarray and real-time RT-PCR.

Study III

In Study III, the expression of P2 receptors in human umbilical veins exposed to shear stress was investigated. Vessels were stimulated in the *ex vivo* vascular perfusion system and both endothelium and smooth muscle cells were isolated and analyzed with real-time RT-PCR, Western blotting and immunohistochemistry.

Study IV

Study IV was designed to examine the interplay between shear stress and inflammatory stress on u-PA and PAI-1 expression. Cultured human umbilical vein endothelial cells (HUVECs) were exposed to shear stress in an *in vitro* shear stress stimulation device and TNF-α was added to the perfusion medium. Gene expression was analyzed by real-time RT-PCR and protein levels were measured by enzyme-linked immunosorbent assay (ELISA). Gel shift analysis was used to study potential DNA-nuclear protein interactions in the u-PA enhancer.

The *ex vivo* vascular perfusion model

The vascular perfusion system has previously been described [115]. A schematic of the perfusion system is shown in Figure 2. Briefly, the perfusion circuits are driven by hydrostatic pressure created by the vertical distance between up- and down-stream reservoirs. Each vessel segment is mounted in a perfusion chamber, which is placed in a 37˚C water bath. Perfusion medium (Tyrodes solution, pH 7.4) is constantly pumped through a peristaltic pump and a heat exchanger is utilized to ensure a constant temperature of 37 ± 0.1˚C in the perfusion medium. The perfusion system is operated by a computerized control and feedback system. Data from up- and down-stream pressure transducers and an electromagnetic flow detector are recorded and digitized through a

data acquisition board. Digital signals are processed in a Macintosh Power PC Computer 7600/120 MHz, equipped with a custom-assembled program developed by our group in LABVIEW 4.0.

Shear stress is calculated by the formula:

$$\tau = \frac{1}{2}\left(\frac{\Delta P}{L}\right)^{\frac{3}{4}} \cdot \left(\frac{8\eta Q}{\pi}\right)^{\frac{1}{4}}$$

where τ is wall shear stress, ΔP is the pressure drop over the vessel, L is the vessel length, η viscosity of the fluid, and Q is the flow through the vessel. Through computerized control of the height regulator and the proportionating solenoid valve, various combinations of hydrodynamic perfusion parameters can be generated. The software permits continuous real-time monitoring of perfusion pressure (P1, P2), mean intraluminal pressure [(P1+P2)/2], flow rate, pH value, shear stress, vascular resistance (defined as pressure drop/flow rate) and Reynold´s number. pH of the perfusion medium is kept constant by controlled gas bubbling.

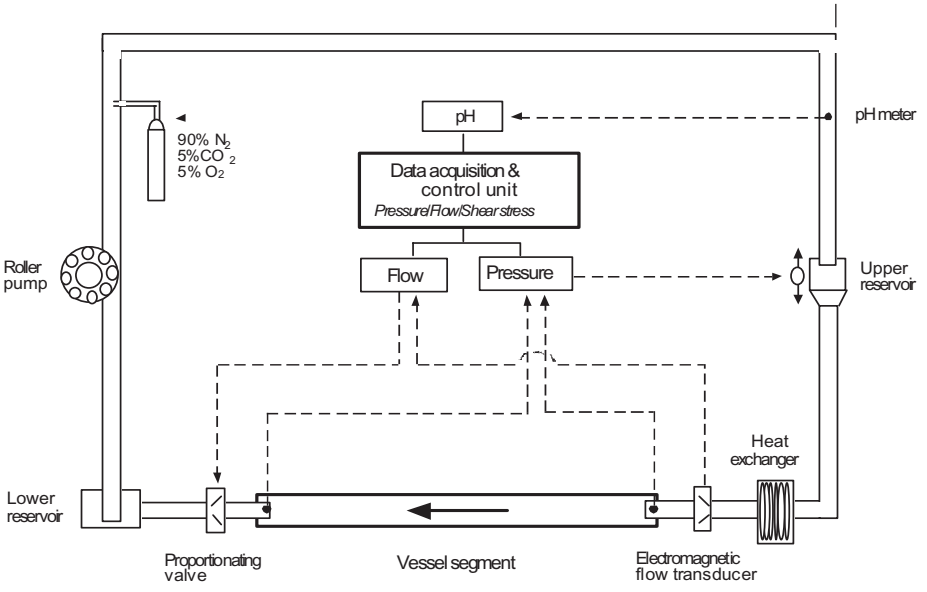


Figure 2. Schematic of the perfusion system.

Ex vivo vessel preparation procedure

Umbilical cords were obtained immediately after delivery from single, vaginal deliveries at the maternity ward at Sahlgrenska University Hospital/Östra. The umbilical cord was divided into two parts, one used for each circuit. Both vessel segments were carefully cannulated and rinsed with phosphate buffered saline (PBS) to remove any remaining blood. Placental and fetal segments were randomized to normal/low and high circuits to eliminate any systemic variation due to the differences between the

two vessel segments. The umbilical veins were kept *in situ i.e.* not dissected free from the surrounding Wharton´s jelly. All veins were perfused from the placental to the fetal end, *i.e.* in the same direction as *in vivo*.

The experiments were performed in parallel on vessel pairs, and served thereby as its own control. Each segment was approximately 20 cm. Prepared vessels were mounted in the organ perfusion chamber and connected to the fluid loops of the perfusion system. After a 10-min non-recirculating washout period, vessels were equilibrated for another 30 min under constant mean intraluminal pressure and flow rate of 20 mmHg and 10 ml/min, respectively. Thereafter, the target shear stress level was set to 10 dyn/cm^2 in both circuits, and intraluminal pressure level was set to 40 mmHg (high pressure) or 20 mmHg (normal pressure) in respective circuits for pressure experiment. For shear stress experiment the intraluminal level was set to 20 mmHg in both circuits, and shear stress to 25 dyn/cm^2 (high shear stress) and <4 dyn/cm^2 (low shear stress). After 6h perfusion, endothelial cells were eluted by incubating each vessel with 0.1% collagenase for 12 min at 37˚C. The endothelial cells were rinsed out with PBS and the cell suspension was then centrifuged at 260g. The smooth muscle layers were obtained by dissecting them free of adhering tissue and then homogenized for further RNA and protein extraction.

Cell culture

Fresh umbilical cords were obtained from the maternity ward, Sahlgrenska University Hospital/Östra, and HUVECs were isolated by collagenase (Sigma-Aldrich, St Louis, MO, USA) digestion [129]. Human aortic endothelial cells (HAECs) were purchased from Clonetics (Cambrex). Cells were incubated at 37˚C in a humidified 5% CO_2 incubator and maintained in EGM-2 complete culture medium, containing EBM-2 basal medium supplemented with 2% fetal bovine serum and growth factors (SingleQuots®, Clonetics/Cambrex). Subcultures were obtained by trypsin/EDTA (Sigma-Aldrich) treatment of confluent monolayers. HUVECs were used in passage 1 and HAECs in passage 5.

In vitro shear stress stimulation model

Flow experiments with cultured HUVECs were performed with the Streamer™ shear stress device (Flexcell International). The device is a parallel-plate flow system that enables stimulation of cultured cell with fluid-induced laminar shear stress. HUVECs were seeded onto fibronectin coated (Roche Diagnostics) culture slips® (Flexcell International Corporation) and grown for 48-72h to confluence. The culture slides were mounted in two Streamer™ chambers for simultaneous stimulation with either low (1.5 dyn/cm^2) or high (25 dyn/cm^2) laminar shear stress. Each Streamer chamber was incorporated into a recirculating loop fed with perfusion medium (50% EGM-2, 50% M199 (Cambrex), total 2% fetal bovine serum). Each loop was driven by a peristaltic roller pump equipped with a pulse dampener. The components were connected by silicone rubber tubing and both systems were placed in a 37°C humidified 5% CO_2 incubator. Cells were exposed to high or low shear stress for 6 and 24h for mRNA

quantification and 3 and 24h for electrophoretic mobility shift assay (EMSA) experiments. To study effects of combined inflammation and shear stress stimulation, TNF-α (1 ng/ml) was added to the perfusion media after two hours of pre-shear stimulation and continued for up to 24h.

Stimulation with proinflammatory cytokines

HUVECs were stimulated with 0.1-10 ng/ml of human recombinant TNF-α (Sigma-Aldrich) and 1-100 pg/ml interleukin-1β (IL-1β) (R&D systems) for 6 and 24h. To study cell signaling, HUVECs were incubated with pharmacological inhibitors. Cells were preincubated with the inhibitor 1h prior to addition of TNF-α (1 ng/ml). Ten μM parthenolide (Sigma-Aldrich) were used to inhibit NF-κB signaling, 25 μM SB203580 (Biosource, Nivelles, Belgium) to inhibit p38 MAPK, and 10 μM SP600125 (Calbiochem) to inhibit JNK signaling.

Assay techniques

Real-time RT-PCR

Total RNA was extracted using either Trizol (Invitrogen, Study I, II and III) or E.Z.N.A. total RNA kit, (Omega Bio-Tek, Study IV). Contaminations of DNA were removed by treatment with RNase-Free DNase Set (Omega Bio-Tek, Study IV). RNeasy kit (Qiagen) was used in Study I an II for RNA cleanup. Total RNA concentrations and purity were determined by absorbance measures at 260/280 nm wavelength and RNA quality was controlled on 1% agarose gel. mRNA was converted to cDNA by reverse transcription (GenAmp RNA PCR kit, Applied Biosystems).

Relative quantification of mRNA was performed on an ABI PRISM® 7700 Sequence Detector (Applied Biosystems). The sequence of all used primers and probes are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference gene in Study I, III, and IV to correct for potential variation in RNA loading or efficiencies of the reverse transcription reaction. The principle of the real-time method is that a fluorescently labeled probe hybridizes to its target sequence during PCR, and the Taq polymerase cleaves the reporter dye from the non-extendable probe. The reporter dye is then released to the solution and the increase in dye emission is monitored in real-time. The threshold cycle (C_T) is defined as the cycle number at which the reporter fluorescence reaches a certain level. There is a linear relationship between C_T and the log of the initial target copy number as shown by Higuchi *et al.* [119]. Relative quantification of gene expression was analyzed as a treatment-to-control expression ratio using the comparative C_T method (User Bulletin 2, Applied Biosystems). The relative expression value of the target gene is obtained by calculating the difference in threshold cycles for a target and a reference gene in a treated sample, and comparing it to that of a control sample.

Oligonucleotide primers and TaqMan® probes for quantification of t-PA, PAI-1, vonWillebrand factor (vWF), GAPDH, β-actin, cyclophilin (CYC), hypoxanthine-guanine phosphoribosyltransferase (HPRT), transferrin receptor (TfR), $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2X_1$, $P2X_4$ $P2X_7$, endothelial nitric oxide synthase (eNOS), and

Table 1. Oligonucleotide primers and probes used for real-time RT-PCR

Gene	Oligonucleotide	Sequence
PAI-1	Sense primer	GGC TGA CTT CAC GAG TCT TTC A
	Antisense primer	TTC ACT TTC TGC AGC GCC T
	Probe	ACC AAG AGC CTC TCC ACG TCG CG
t-PA	Sense primer	GGC CTT GTC TCC TTT CTA TTC G
	Antisense primer	AGC GGC TGG ATG GGT ACA G
	Probe	TGA CAT GAG CCT CCT TCA GCC GCT
vWF	Sense primer	GCT TGC TCT GGC CCT CAT T
	Antisense primer	ATG ACC TGC CGC GAG TTC
	Probe	TGC CAG GGA CCC TTT GTG CAG AA
GAPDH	Sense primer	CCA CAT CGC TCA GAC ACC AT
	Antisense primer	CCA GGC GCC CAA TAC G
	Probe	AAG GTG AAG GTC GGA GTC AAC GGA TTT G
β-actin	Sense primer	CGT GCT GCT GAC CGA GG
	Antisense primer	GAA GGT CTC AAA CAT GAT CTG
	Probe	CCT GAA CCC CAA GGC CAA CCG
CYC	Sense primer	GTA CTA TTA GCC ATG GTC AAC CCC
	Antisense primer	CAG TCA AAG GAG ACG CGG CC
	Probe	CGT CGA CGG CGA GCC CTT G
HPRT	Sense primer	GGA CTG ACA CTG GCA AAA CAA TGC A
	Antisense primer	AGC TTG CGA CCT TGA CCA TCT
	Probe	TTG CTT TCC TTG GTC AGG CAG TAT AAT CCA
TfR	Sense primer	AAT CCC AGC AGT TTC TTT CTG TTT C
	Antisense primer	TCC TTA TAG GTG TCC ATG GTG GT
	Probe	TGC GAG GAC ACA GAT TAT CCT TAT TTG GG
$P2Y_1$	Sense primer	CGT GCT GGT GTG GCT CAT T
	Antisense primer	GGA CCC CGG TAC CTG AGT AGA
	Probe	TGG TGG CGA TCT CCC CCA TCC
$P2Y_2$	Sense primer	TTC CGT CCA TTC CAC GTC A
	Antisense primer	TTG AGG GTG TGG CAG CTG A
	Probe	CCC TCT ACT ACT CCT TCC GCT CGC TGG
$P2Y_4$	Sense primer	TGT CCT TTT CCT CAC CTG CAT
	Antisense primer	TGC CCG AAG TGG GTG G
	Probe	CGT GCA CCG CTA CCT GGG CAT C
$P2Y_6$	Sense primer	CCT GCC CAC AGC CAT CTT
	Antisense primer	GGC TGA GGT CAT AGC AGA CAG TG
	Probe	CTG CCA CAG GCA TCC AGC GTA ACC
$P2Y_{11}$	Sense primer	GTT GGT GGC CAG TGG TGT G
	Antisense primer	TTG AGC ACC CGC ATG ATG T
	Probe	CCC TCT ACG CCA GCT CCT ATG TGC C
$P2X_1$	Sense primer	TCT CTC CCC AGG CTT CAA CTT
	Antisense primer	GAG GTG ACG GTA GTT GGT CCC
	Probe	AGG TTT GCC AGG CAC TTT GTG GAG AA
$P2X_4$	Sense primer	CAT CAT CCC CAC TAT GAT CAA CA
	Antisense primer	AGC ACG GTC GCC ATG C
	Probe	CGG CTC TGG CCT GGC ACT GCT A
$P2X_7$	Sense primer	ATC GGC TCA ACC CTC TCC TAC
	Antisense primer	CTG GAG TAA GTG TCG ATG AGG AAG
	Probe	TCG GTC TGG CCG CTG TGT TCA TC
eNOS	Sense primer	CGC AGC GCC GTG AAG
	Antisense primer	ACC ACG TCA TAC TCA TCC ATA CAC
	Probe	CCT CGC TCA TGG GCA CGG TG
VCAM-1	Sense primer	GGA AGA AGC AGA AAG GAA GTG GAA T
	Antisense primer	GAC ACT CTC AGA AGG AAA AGC TGT A
	Probe	CCA AGT TAC TCC AAA AGA C

vascular cell adhesion molecule-1 (VCAM-1) mRNA were designed from the Genbank database using Primer express v1.5 (Applied Biosystems). Each primer pair was selected to ensure that the amplicon spanned an exon junction to avoid amplification of genomic DNA. TaqMan® Gene expression Assay (Applied Biosystems) were used to quantify u-PA (Hs00170182_m1), matrix metalloproteinase-2 (MMP-2, Hs00234422_m1), MMP-9 (Hs00234579_m1), tissue inhibitor of metalloproteinase-1 (TIMP-1, Hs00171558_m1), u-PAR (Hs00182181_m1), tissue factor (Hs00175225_m1), and thrombomodulin (Hs00264920_s1) mRNA levels, whereas VCAM-1 mRNA was quantified with Custom TaqMan® Gene expression Assay (Applied Biosystems) (Table 1). Typically, PCR was carried out in a 25 µl reaction mixture containing; cDNA from 30 ng total RNA, TaqMan® Universal PCR mastermix (Applied Biosystems), 10 pmol of each primer and 5 pmol probe. Samples were analyzed in triplicate or duplicate.

Microarray

RNA (Study I) was pooled into four pools (normal and high pressure, low and high shear stress). The amount of RNA from each experiment was approximately 0.6 µg. Each pool was divided and analyzed on duplicate DNA microarrays according to the following procedure. First, double-stranded cDNA was generated (Life Technologies Superscript Choice system, Life Technologies). Then, labelled cRNA was synthesized from the total amount of cDNA by *in vitro* transcription with biotin-labelled nucleotides and T7 RNA polymerase using the Enzo Bio-Array High Yield RNA Transcript Labeling Kit (Enzo Diagnostics). Labelled cRNA was purified using RNeasy columns (Qiagen) and then fragmented. Gel electrophoresis was performed to verify expected size distribution of cDNA, cRNA and fragmented cRNA. Hybridization cocktails containing fragmented cRNA were prepared according to procedure developed by the manufacturer (Affymetrix).

Two HG U133A arrays (Affymetrix) were hybridized for each experimental condition. In brief, the hybridized probe array was washed and stained with streptavidin phycoerythrin conjugate followed by a signal amplification step performed using biotinylated antistreptavidin antibody. The arrays were scanned and the amount of light emitted, proportional to the amount of bound target at each location on the probe array, was detected.

Scanned output files were visually inspected for hybridization artifacts and then analyzed with Microarray suite 5 (MAS 5, Affymetrix). To allow comparison of gene expression, the arrays were globally scaled to an average intensity of 100. RNA expression was quantitatively estimated using the signal algorithm (Affymetrix), which calculates the difference in signal between a set of oligonucleotides that perfectly matched the gene and a set of mismatch control oligonucleotides. In addition, a qualitative estimate of gene expression was given by the Detection Call. This was obtained by an algorithm based on the signal intensity and quality of the average difference (Affymetrix), in which the Detection Call gene expression is classified as absent, marginal, or present.

Comparisons were made between the results from the duplicate DNA microarrays used for analysis of the endothelium exposed to normal pressure and the duplicate DNA microarrays used for analysis of the high pressure exposed endothelium, generating a total of 4 comparisons. Genes with different expression levels when exposed to normal or high pressure were identified by the Change Call algorithm (Affymetrix). With the Change Call, a gene is classified as increased, marginally increased, no change, marginally decreased, or decreased. Genes having a Change Call of increased, marginally increased, or decreased, marginally decreased in 3 or 4 of the comparisons were classified as regulated [130]. Comparison of the results of the duplicate DNA microarrays from the shear-exposed endothelium was made in the same manner as for the pressure DNA microarrays. A signal ratio was also calculated by using the mean signal of the duplicated DNA microarrays.

Genes were classified into functional groups according to the Proteome database [131]. The annotation systems of Organismal role and Cellular role were used. Cellular and Organismal role annotations were available in the database for 18% and 26% of the regulated genes, respectively.

SDS-PAGE and Western blotting

Protein electrophoresis was performed on 10% Tris-HCl polyacrylamide ready gels (Bio-Rad Laboratories) and electroblotted onto nitrocellulose membranes (Hybond-C extra, Amersham Pharmacia Biotech). Protein loading was 10 μg for each well. After transfer, the membrane was blocked for 1h in tris buffered saline (TBS) containing 0.1% Tween 20 and 5% dried skimmed milk to minimize non-specific binding. Thereafter, membranes were incubated with the following primary antibodies; anti-$P2Y_1$ (1:400, Alomone Labs), anti-$P2Y_2$ (1:200, Alomone Labs) anti-$P2Y_6$ (1:250, Alomone Labs), and anti-$P2X_1$ (1:250, GlaxoSmithKline Research & Development) overnight. A negative control with a peptide antigen preincubated with the same amount of antibody for 1h at room temperature was also included. Then, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:1500, anti-rabbit IgG) for 1h. Proteins were visualized by the ECL™ Western blotting RPN 2108 system (Amersham Pharmacia Biotech) and signals were detected by autoradiography. Membranes were reprobed with an anti-GAPDH antibody (1:20 000, Chemicon International) as control. The immunoreactive band densities were quantified with a scanner and Quantity One® software. Within this software, volume background subtraction was used, which was the volume of the band minus the volume of the same area of background. Thus, greater band values reflect darker and/or larger bands. The optimized band value was calculated from the value of the P2 receptor divided by the value of GAPDH, which was obtained from the same blot membrane.

Immunohistochemistry

In Study III, 10-mm vascular segments were cut from the middle part of each umbilical cord after perfusion and fixed in 4% formalin, and then embedded in paraffin. Five-micron paraffin sections were cut and mounted on glass slides. Each matched

vessel pair was placed on the same slide and stained at the same time and under the same conditions. The avidin-biotin-peroxidase complex method was used. After incubation with normal serum, the sections were incubated with anti-$P2Y_1$ (1:200), anti-$P2Y_2$ (1:100), anti-$P2Y_6$ (1:100) and anti-$P2X_1$ (diluted 1:100) antibodies and negative control (peptide antigen preincubated with antibody). Bound primary antibody was detected using VECTASTAIN® Elite ABC kit and developed with DAB substrate kit for peroxidase. After counterstaining with VECTOR® hematoxylin QS nuclear counterstain (Modified Mayer's Formula), the slides were examined by light microscopy.

Enzyme-linked immunosorbent assay (ELISA)

Concentrations of u-PA antigen in the cell culture medium and intracellular levels of u-PA antigen (Study IV) were determined using ELISA ZYMOTEST (Haemochrom Diagnostica). The principle of this assay is that samples or standard containing human recombinant protein, are added to microtest wells that are coated with anti-u-PA IgG. After u-PA has been allowed to bind to the antibodies, peroxidase-labelled anti-u-PA IgG is added. Peroxidase then converts the substrate to a yellow product that is directly proportional to the amount of protein present in the sample. The sample concentrations were obtained by spectrophotometric quantification.

Electrophoretic mobility shift assay (EMSA)

EMSA was used to detect interactions between nuclear proteins and regulatory elements in the u-PA enhancer (Study IV). Double-stranded oligonucleotides were designed to contain two specific AP-1 elements, PEA3/AP-1a (tgtccaggaggaaatgaagtcatctg) and AP-1b (gagcaacatgaatcatgacg) in the u-PA enhancer. Labeling was performed using T4 polynucleotide kinase (USB) and [γ^{32}P] ATP (adenosine triphosphate) (Amersham Bioscencies) [132]. Annealing was performed by adding a molar excess of the complementary strand to the kinase treated mixture which was then heated to 100˚C, after which the probes were left to anneal during the cooling–down process. Probes were gel-purified by electrophoresis through 12% native polyacrylamide gels, visualized by autoradiography, excised and eluted in a buffer containing 0.5 M ammonium acetate and 1mM EDTA. Labeled oligomer were precipitated with ethanol and resuspended in NaCl/Tris/EDTA buffer.

Preparation of nuclear extracts from HUVEC was performed as previously described [133] and concentrations were quantified using Bio-Rad Protein Assay. For each reaction, 2.5 µg nuclear protein extract in Osborne buffer D was preincubated with poly[d(I-C)], SMK buffer (12 mM spermidine, 12 mM $MgCl_2$ and 200 mM KCl) and ^{32}P-labeled probe as described (Study IV). Nuclear protein and oligonucleotide complexes were separated by electrophoresis in a native 5% polyacrylamide gel, and visualized by autoradiography. To identify specific proteins involved in DNA-binding, supershift experiments were performed using antibodies (Santa Cruz Biotech, Santa Cruz, CA, USA) against c-jun (#sc-1694), c-fos (#sc-52), and activating transcription factor-2 (ATF-2) (#sc-187). Supershift experiments were performed according to the

same procedure as that described for standard EMSAs, except that 2 μg specific antibody was added to the nuclear extracts at the same time as the labeled oligonucleotide. The extracts were then incubated with probe and antibody for 1h on ice before being loaded on the gel.

Statistics

Data are presented as mean and standard error of the mean (Study I, III and IV) or standard deviation (Study II). Paired Student's *t*-test was used for statistical evaluation in all studies. ANOVA (analysis of variance) was also used in Study III. Correlation coefficient and regression equations were computed by Spearman regression analysis in Study II. A p-value <0.05 was considered significant.

RESULTS

Global gene expression response patterns of biomechanically exposed endothelium (Study I)

DNA microarray expression profiling was performed on endothelium stimulated with either pressure or shear stress. The normal pressure protocol was assumed to represent the physiological condition in the vessel regarding levels of intraluminal pressure and shear stress. Of the 22,283 genes included on the HG U133A Affymetrix microarray chips, 9,065 genes (41%) were detectable according to the Affymetrix detection algorithm in both duplicates of the normal pressure DNA microarray. Another 1,871 genes were expressed in one or more of the duplicates of the arrays from vessels perfused under high-pressure perfusion or high- or low-shear stress perfusion. Together these genes were 10,936 (49%), which were considered expressed in the vascular endothelium of the umbilical vein. Regulated genes were identified by scoring the result of the Affymetrix change call algorithm. A total of 1,825 genes were found to be either induced or suppressed by pressure or shear stimulation, or both. This means that 17% of all genes expressed in the vascular endothelium were responsive to mechanical stimulation. Following pressure stimulation, 647 genes were induced and 519 genes were suppressed. Shear stress induced 133 genes and suppressed 771 genes.

Seven different genes involved in hemostasis were validated with quantitative real-time RT-PCR. Tissue factor mRNA was highly down-regulated by pressure and shear in the microarray analysis, and the suppression was confirmed with real-time RT-PCR (pressure $p<0.01$ and shear stress $p<0.01$). PAI-1 was slightly down-regulated by pressure in the microarray analysis, which could be confirmed by real-time RT-PCR ($p<0.01$). PAI-1 was not regulated by shear stress in the microarray analysis, but was slightly suppressed when analyzed with real-time RT-PCR ($p=0.05$). t-PA was not regulated by any of the two stimuli in the microarray analysis. However, the real-time RT-PCR analysis showed a small down-regulation by pressure ($p=0.05$), but no change by shear stress. Thrombomodulin was induced by pressure in the microarray analysis. A similar response was seen in the real-time RT-PCR, but it fell short of statistical significance. No change of thrombomodulin by shear stress was observed in the microarray or real-time RT-PCR analyses. u-PA was down-regulated by pressure in the microarray analysis, which could not be confirmed by real-time RT-PCR. u-PA was not regulated by shear stress with any of the two methods used. u-PAR was suppressed by both pressure and shear stress in the microarray analysis, which was also observed with real-time RT-PCR (pressure $p=0.11$ and shear stress $p<0.02$). vWF was neither regulated by the two stimuli in the microarray analysis nor in the real-time RT-PCR.

Although the majority of genes were only regulated by one of the two mechanical stimuli, a total of 245 genes were found to be regulated by both shear stress and pressure. On average, only one out of eight regulated genes was responsive to both mechanical stimuli. A scatter plot of the distribution of the genes regulated in the same or opposite direction by shear and pressure is shown in Figure 3. Interestingly, 210 of the 245 genes were down-regulated by both stimuli.

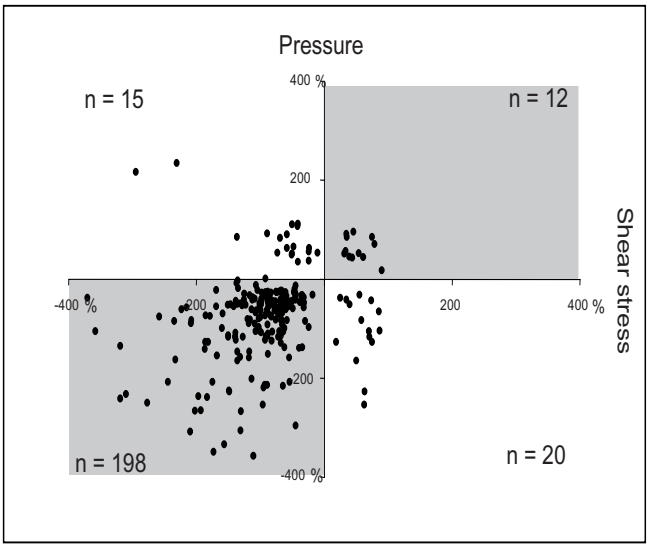


Figure 3. The distribution of the overlapping genes that responded to both pressure and shear stress. Shaded area indicates genes that responded in the same direction by the two stimuli. Open area indicates genes that are regulated in opposite direction by the two forces. The relative change of expression of the overlapping genes are expressed in percent. 100% indicate a 2-fold increase.

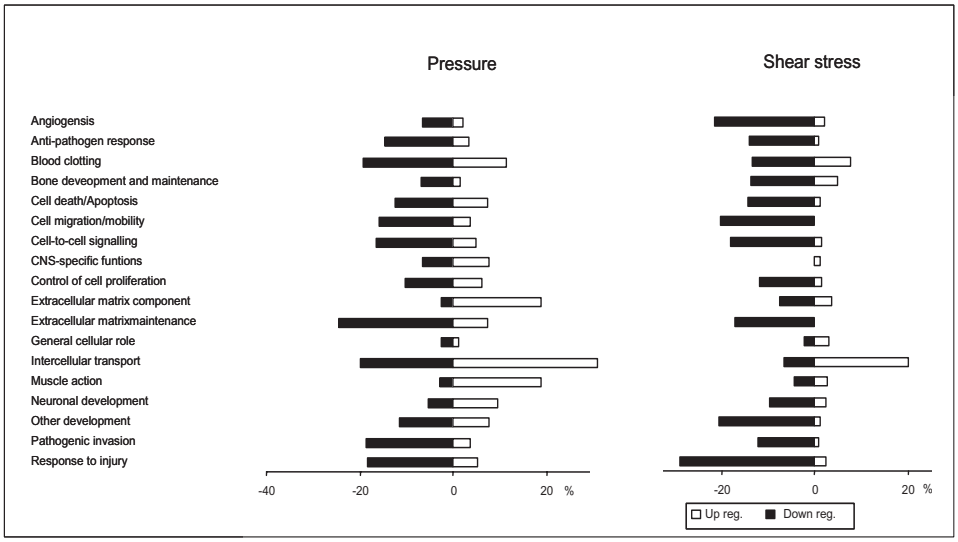
Among the genes with the highest signal ratios, Intracellular adhesion molecule 4 was up-regulated four times and u-PA down-regulated five times by pressure. Krüppel-like factor 2 was induced by both shear and pressure, whereas MMP-1, and MMP-10 were suppressed by both stimuli.

To further explore the hypothesis that the two different biomechanical forces induce distinct gene expression patterns, all regulated genes were classified into functional groups according to their assigned organismal and cellular roles as defined in the Proteome database (Figure 4 and 5). This analysis showed that for a number of functional groups, the response patterns induced by shear and pressure were distinctly different. Functional groups with highly differentiated responses included genes involved in extracellular matrix component, angiogenesis, control of cell proliferation, chromatin/chromosome structure, nuclear-cytoplasmic transport, and DNA repair.

Influence of biomechanical stimulation on expression of potential reference genes (Study II)

Table 2 shows the signal ratio of ten reference genes analyzed by microarray. The genes were selected from different functional classes in order to reduce the risk that they might be co-regulated in an experimental situation. The TfR gene was the gene

Organismal role



Cellular role

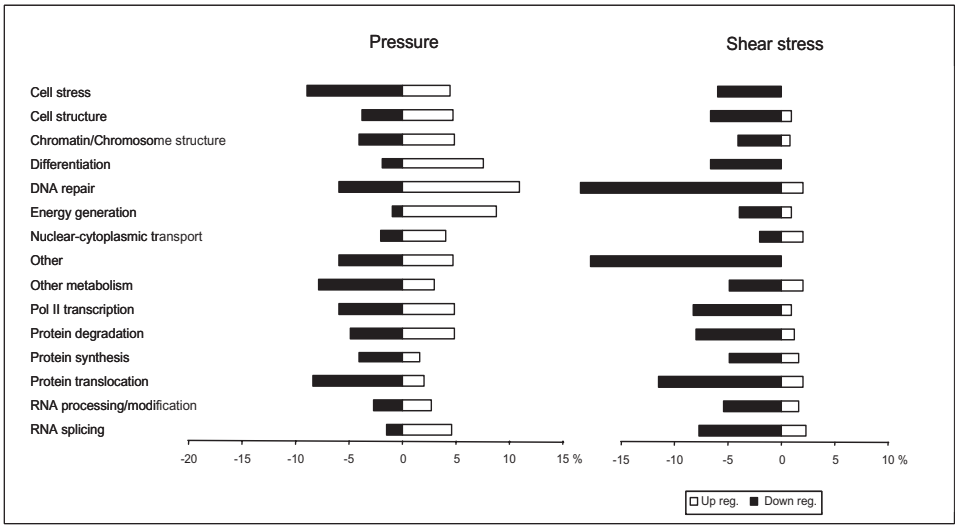


Figure 4. Functional groups according to the Proteome databases “Organismal role” annotation system. Groups with less than 16 annotations are not shown in the figure. Left panel shows genes regulated by pressure and right panel shows genes regulated by shear stress. Closed bars indicate down-regulated genes and open bars indicate up-regulated genes.

Figure 5. Functional groups according to the Proteome databases “Cellular role” annotation system. Groups with less than 16 annotations are not shown in the figure. Left panel shows genes regulated by pressure and right panel shows genes regulated by shear stress. Closed bars indicate down-regulated genes and open bars indicate up-regulated genes.

Table 2. Reference genes evaluated from the microarray data

Reference gene	Shear stress signal ratio	Pressure signal ratio
CYC	1.2	0.9
β-actin	1.2	0.9
GAPDH	1.1	0.9
	1.3	0.8
	1.2	0.8
HPRT	1.0	1.1
TfR	0.7	0.6
B2M	1.1	0.8
	1.1	1.0
HMBS	Not detected	
SDHA	1.7	1.0
	1.1	1.1
TBP	Not detected	
UBC	1.2	0.8

Abbreviations: B2M denotes Beta-2-microglobulin, HMBS Hydroxymethylbilane synthase, TBP TATA-box binding protein, UBC Ubiquitin C. Note. GAPDH, B2M, and SDHA were represented by more than one probe set on the microarray.

with the greatest down-regulation in both shear stress and pressure experiments. The SDHA gene had a fold change of 1.7 in response to shear stress. Hydroxymethylbilane synthase and TATA-box binding protein were not detected in the baseline situation. All other genes analyzed from microarray data were not considered significantly regulated when applying Affymetrix The Change call algorithm and ratios varied between 0.8 and 1.3.

Further, five of the reference genes were analyzed with real-time RT-PCR. Table 3 and 4 show the C_T values for the reference genes in vessels exposed to high/low shear stress or pressure. Shear stress did not induce any significant change in expression of any of the five reference genes. For pressure stimulation, only TfR showed decreased expression in response to high pressure ($p=0.04$). However, although the average expression of the five reference genes was in most cases independent of the biomechanical stimulation, the individual high-versus low ΔC_T comparison showed substantial scatter. As shown in Table 3 and 4, the standard deviations of the ΔC_T high-low values varied between 1.2 and 4.2 cycles. Under pressure experimental conditions, β-actin, CYC, and HPRT showed the lowest variations both in absolute and relative terms. For shear experiments, β-actin, GAPDH and CYC had the lowest variation combined with very low average ΔC_T high-low differences. By contrast, TfR showed a considerable variation between shear conditions. In general, the between-condition variations were somewhat lower in the pressure than the shear stress series.

Table 3. C_T values for the five reference genes in vessel segments exposed to high or low shear stress, and paired comparisons of ΔC_T values (segment exposed to high minus segment exposed to low shear) in absolute numbers and in percent relative to average absolute C_T level. n=11

	High shear stress	**Low shear stress**	**Paired difference of high vs low ΔC_T values**	**Coefficient of variation for paired difference**	**t-test**
	Mean ± SD	Mean ± SD	Mean ± SD	%	p
GAPDH	25.5 ± 3.2	25.4 ± 3.3	0.09 ± 2.02	7.9	0.88
β-actin	25.9 ± 1.7	26.0 ± 2.6	-0.05 ± 1.78	6.9	0.59
CYC	27.9 ± 1.9	27.7 ± 2.0	0.18 ± 2.26	8.2	0.79
HPRT	31.2 ± 1.7	30.8 ± 1.4	0.40 ± 2.54	8.2	0.61
TfR	31.4 ± 2.8	31.1 ± 4.7	0.28 ± 4.23	13.5	0.84

Table 4. C_T values for the five reference genes in vessel segments exposed to high or low intraluminal pressure, and paired comparisons of ΔC_T values (segment exposed to high minus segment exposed to low pressure) in absolute numbers and in per cent relative to average absolute C_T level. n=10

	High pressure	**Low pressure**	**Paired difference of high vs low ΔC_T values**	**Coefficient of variation for paired difference**	**t-test**
	Mean ± SD	Mean ± SD	Mean ± SD	%	p
GAPDH	25.7 ± 1.6	25.3 ± 2.1	0.36 ± 2.34	9.3	0.65
β-actin	25.7 ± 1.2	25.4 ± 1.6	0.26 ± 1.17	4.6	0.50
CYC	28.3 ± 1.2	27.9 ± 1.7	0.43 ± 1.55	5.5	0.40
HPRT	32.1 ± 2.2	31.8 ± 1.7	0.26 ± 1.24	3.9	0.53
TfR	32.6 ± 1.6	31.3 ± 1.3	1.25 ± 1.55	4.9	0.04

The variability among conditions is further illustrated in Figure 6, which shows the individual ΔC_T high-low differences vessel by vessel. This histogram shows different patterns of variability. For instance vessel number 1 in the shear experiments had very variable ΔC_T high-low among the genes, vessel 11 had constantly high and positive ΔC_T high-low in all five genes, which may indicate that the latter difference is due to variability outside the PCR protocol.

The tube-to-tube variations were extremely low for all five reference genes. Variations coefficients for C_T values of triplicate reactions were below 1%. To estimate the variation induced by differences in efficiency of the reverse transcription step, five separate cDNA synthesis procedures were performed. Variation coefficients for the variability among repeated reverse transcriptions were for GAPDH 2.1%, β-actin 2.1%, CYC 2.5%, HPRT 2.3%, and TfR 2.4%.

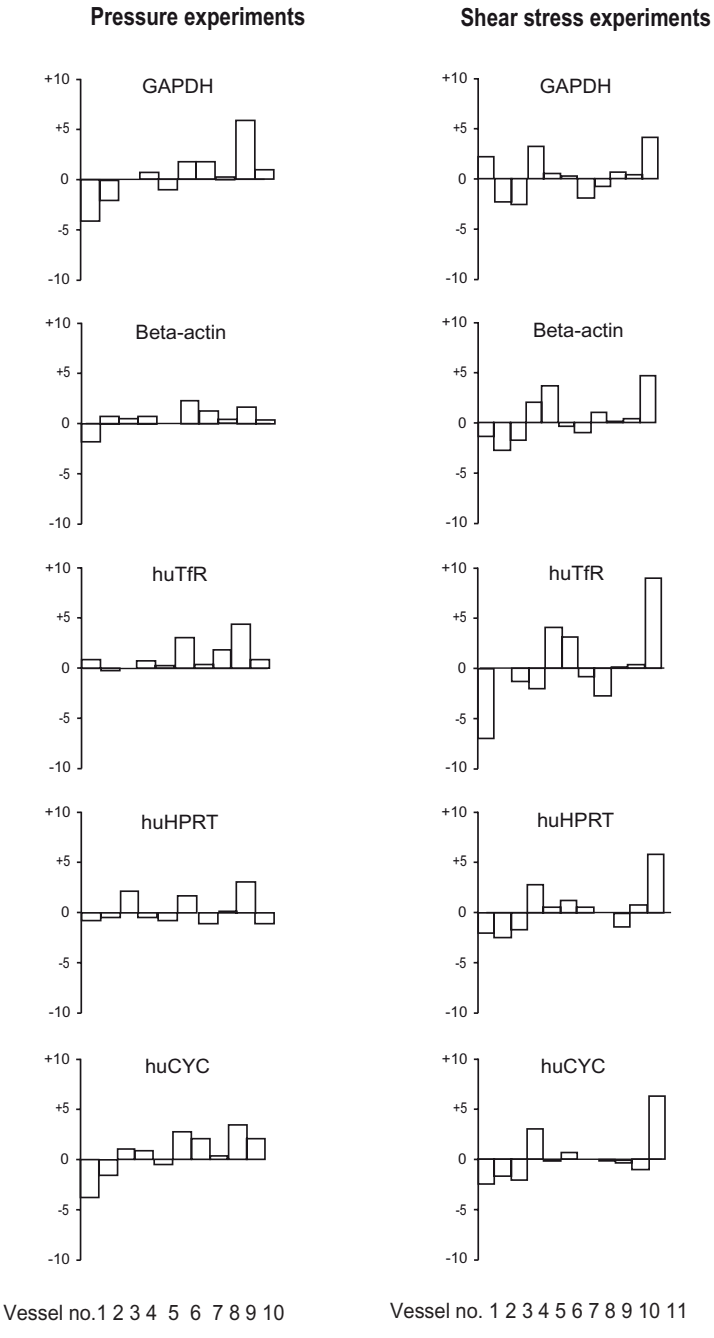


Figure 6. Bar graphs illustrating individual ΔC_T high-low comparisons of each pair of vessel segments exposed to high or low intraluminal pressure (left panel) and high or low shear stress (right panel).

Effects of shear stress on P2 receptors in smooth muscle cells (Study III)

The regulatory effects of shear stress on the gene expression of P2 receptors in endothelial and smooth muscle cells were examined. Compared to low shear stress, high shear stress reduced mRNA level of $P2X_1$, whereas, $P2Y_2$, and $P2Y_6$ levels were induced (Figure 7, $p<0.05$) in SMCs after 6h. $P2X_1$ was the most highly expressed P2 receptor in SMCs. No significant changes were observed in transcript levels of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2X_4$, and $P2X_7$ in the endothelium.

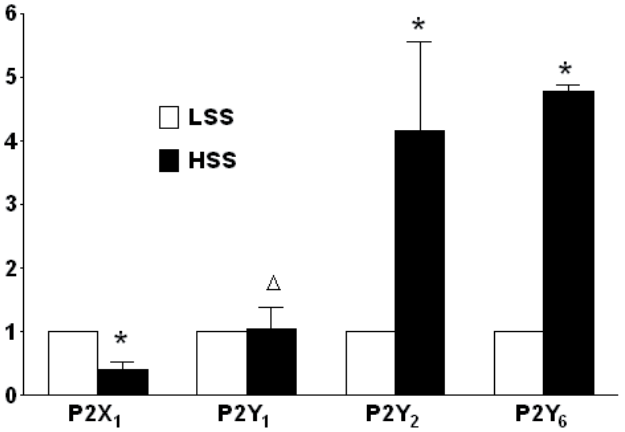


Figure 7. Relative mRNA expression of $P2X_1$, $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors in smooth muscle cells after 6h perfusion. Human umbilical veins were exposed to high (25 dyn/cm^2) or low shear stress (<4 dyn/cm^2) at identical mean perfusion pressure (20 mmHg). n=6, *p<0.05; Δ: p>0.05.

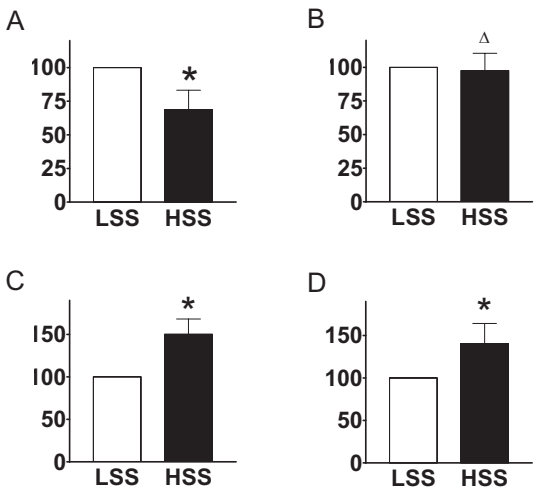


Figure 8. Western blot analysis demonstrating the effect of shear stress on A. $P2X_1$. B. $P2Y_1$ C. $P2Y_2$, and D. $P2Y_6$ protein level in smooth muscle cells. The bars represent the value of relative intensity of the receptor blot band compared to GAPDH. n=5, *p<0.05.

Protein levels of P2 receptors in SMCs exposed to low or high shear stress were analyzed with Western blotting. The 35-kDa GAPDH band was detected in parallel to verify the amount of protein loaded and to calibrate integration of the protein bands. The distribution of $P2X_1$ receptors in shear-exposed SMCs, relative to GAPDH is shown in Figure 8A. The intensity of the band was lower in cells exposed to high shear stress compared to low ($p<0.05$). Similar to mRNA data, no difference in $P2Y_1$ distribution between high and low shear stress was observed with Western blotting (Figure 8B). The $P2Y_2$ and $P2Y_6$ receptors showed stronger bands under high shear stress compared to low shear stress. The relative distribution compared to GAPDH is shown in Figure 8C and 8D.

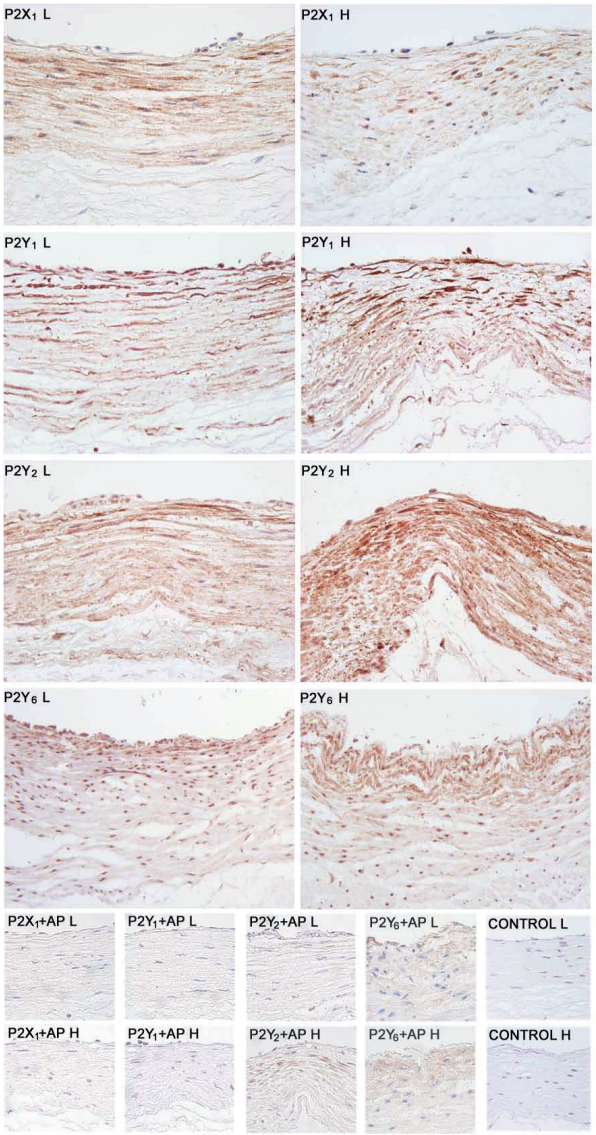


Figure 9. P2 receptor expressions as revealed by immunohistochemistry. P2 receptor expressions in human umbilical veins exposed to high (H) and low (L) shear stress. Brown (diaminobenzidine substrate) was positive staining color. Controls with antigenic peptides (AP) of each receptor and controls without primary antibodies (control) are shown. n=6.

Positive immunoreactivity was observed for $P2X_1$, $P2Y_1$, $P2Y_2$, and $P2Y_6$ (Figure 9). $P2X_1$ staining was only found in the SMC layer, whereas $P2Y_1$, $P2Y_2$, and $P2Y_6$ were found in both endothelium and smooth muscle cell layers. SMCs had a stronger staining beneath the internal elastic lamina than at a distance from it. In high compared to low shear stress sections, the staining intensity in SMCs was decreased for the $P2X_1$ receptor, increased for $P2Y_2$ and $P2Y_6$ receptor, and similar for $P2Y_1$ receptor. The staining patterns were similar in all vessel pairs.

The interplay between shear stress and inflammation on the u-PA expression (Study IV)

High laminar shear stress reduced u-PA mRNA expression in HUVECs after 24h, both in comparison with static control cells and those exposed to low shear stress (Figure 10) ($p<0.001$). A suppression of u-PA transcription level in high shear stress was also observed in HAECs. No significant change of low or high shear stress stimulation was observed in HUVECs or HAECs after 6h.

Next, we examined the regulation of shear stress on the gene expression of PAI-1, the main inhibitor of u-PA. Compared to static control cells, both low and high shear stress induced PAI-1 mRNA expression approximately 2-3 fold at 6 and 24h ($p<0.05$). The effect of shear stress on the gene expression of the u-PA receptor, u-PAR, was investigated and showed a transient induction after 6h, but no significant change was observed after 24h.

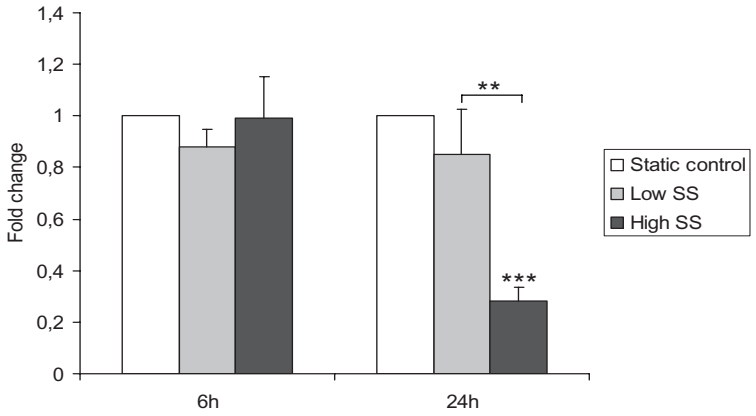


Figure 10. Relative mRNA expression of u-PA in HUVECs exposed to low (1.5 dyn/cm2) or high (25 dyn/cm2) laminar shear stress for 6 or 24h (n=8). Unless indicated in the figure, statistical comparisons are made relative to static controls. **p<0.01 and ***p<0.001.

u-PA gene expression has previously been reported to be induced by proinflammatory cytokines, such as TNF-α and IL-1β [97, 107]. In order to investigate the hypothesis that shear stress could act protectively against inflammation, the effect of TNF-α and IL-1β on u-PA expression was first confirmed. u-PA mRNA levels were dose-dependently increased at both 6 and 24h when HUVECs were exposed to 0.1, 1.0, and 10 ng/ml TNF-α (Figure 11). An approximately 2-fold increase on protein level was also found when HUVECs were exposed to 1.0 ng/ml TNF-α for 6 and 24h. u-PA mRNA was elevated with IL-1β treatment, but the induction was less pronounced than with TNF-α.

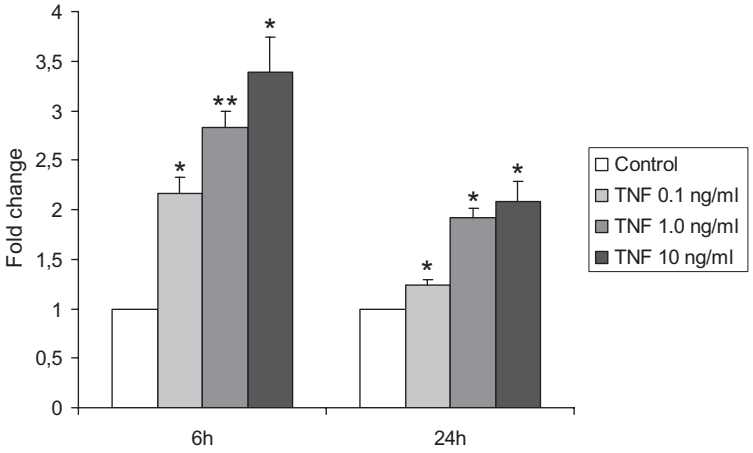


Figure 11. Relative mRNA expression of u-PA in HUVECs stimulated with 0.1, 1.0 or 10 ng /ml TNF-α for 6 and 24h (n=3). Statistical comparisons are made relative to untreated control cells, *p<0.05 and **p<0.01.

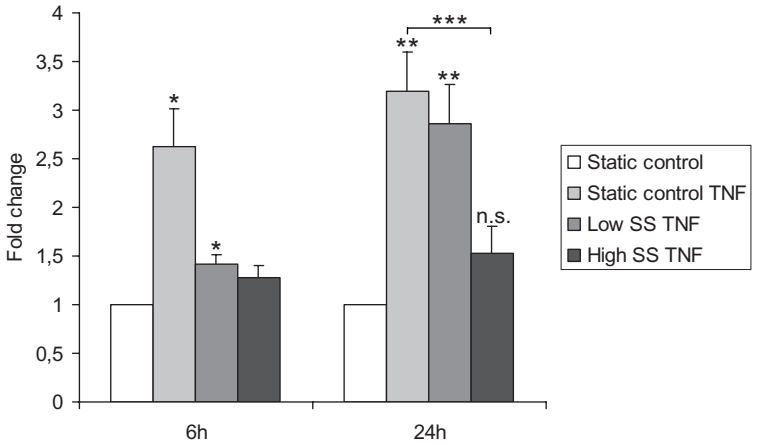


Figure 12. Relative mRNA expression of u-PA in HUVECs exposed to shear stress and TNF-α stimulation. Cells were exposed to low (1.5 dyn/cm^2) or high (25 dyn/cm^2) laminar shear stress and TNF-α (1 ng/ml) for 6h (n=4) or 24h (n=8). Unless indicated in the figure, statistical comparisons are made relative to untreated static control cells, *p<0.05, **p<0.01, and ***p<0.001.

HUVECs were then simultaneously exposed to shear stress and TNF-α for 6 and 24h (Figure 12). u-PA expression was slightly increased in low-sheared TNF-α treated cells after 6h, compared to untreated static cells. Interestingly, after 24h, the u-PA transcript level in high-sheared TNF-α stimulated cells was not significantly changed compared to static cells without TNF-α treatment. On the other hand, u-PA expression in TNF-α exposed low-sheared cells did not differ from TNF-α exposed static cells. This suggests that shear stress has a protective effect on TNF-α induced u-PA expression.

PAI-1 expression was induced 2-3 fold in static TNF-α stimulated HUVECs. Simultaneous shear stress and TNF-α stimulation additively induced PAI-1 expression resulting in a 5-6 fold induction in both high and low shear, compared to untreated static control cells (Figure 13).

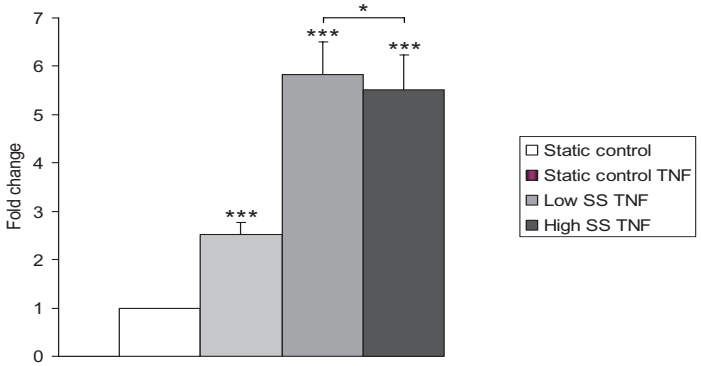


Figure 13. Relative mRNA expression of PAI-1 after 24h in HUVECs exposed to shear stress and TNF-α stimulation. Cells were exposed to low (1.5 dyn/cm^2) or high (25 dyn/cm^2 laminar shear stress and TNF-α (1 ng/ml) for 24h (n=8). Unless indicated in the figure, statistical comparisons are made relative to untreated static control cells, *p<0.05, and ***p<0.001.

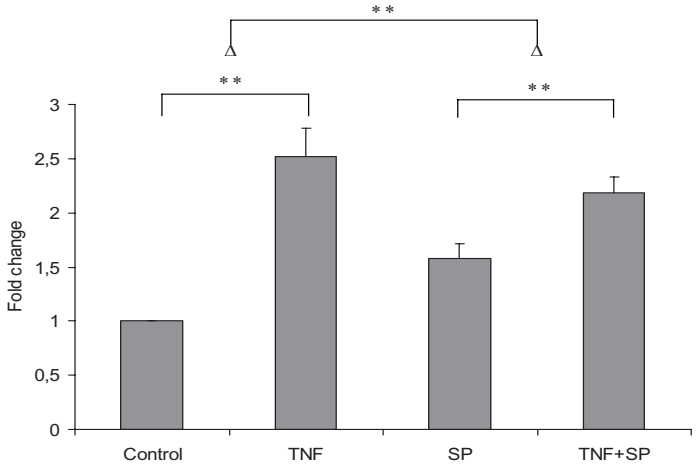


Figure 14. Relative mRNA expression of u-PA in HUVECs exposed toTNF-α (1 ng/ml) for 24h. Effects of inhibition of the JNK pathway with 10 μM SP600125. Statistical comparisons are indicated in the figure. Δ indicates relative difference between TNF-α stimulated cells and control cells or TNF-α and SP treated cells compared to SP treated cells. n=6 **p<0.01.

To validate the experimental set-up, responses of two well-documented shear-responsive genes (VCAM-1 and eNOS) were analyzed. The results were in agreement with the anticipated dose-dependent reduction of VCAM-1 and the induction of eNOS expression. Further, TNF-α is known to modulate gene expression of both VCAM-1 and eNOS, with an induction of VCAM-1 and a reduction of eNOS, which was confirmed in our experiments. Interestingly, when combining the two stimuli, shear stress counteracted the TNF-α mediated induction of VCAM-1 gene expression. Addition of shear stress to TNF-α stimulated cells reduced the VCAM-1 expression by 81% in high shear stress and 66% in low shear stress, respectively, compared to static TNF-α stimulated cells. By contrast, high shear stress significantly prevented the TNF-α mediated reduction of eNOS in TNF-α stimulated cells, compared to TNF-α stimulated static cells.

Pharmacological inhibitors against three major inflammatory signaling pathways activated by TNF-α in endothelial cells [134, 135] were used to investigate how TNF-α induced u-PA expression is mediated. The different inhibitors acted on the NF-κB, the p38 MAPK, and the JNK pathways. The induction was partially inhibited when cells were treated with the JNK inhibitor SP600125, which reduced the relative TNF-α induction by 50% compared to control cells (Figure 14). However, no inhibition of the induction was observed with the NF-κB inhibitor or the p38 MAPK inhibitor, indicating that these pathways were of less importance for the induced u-PA expression.

In order to further investigate potential transcription binding elements in the u-PA enhancer mediating the effect of TNF-α, gel shift analysis was preformed. Nuclear extracts from endothelial cell treated with TNF-α were incubated with probes containing the two AP-1 elements in the u-PA enhancer. Treatment of cells resulted in enhanced interaction with both AP-1b (Figure 15A) and PEA3/AP-1a probes. However, the enhanced binding was less pronounced with the PEA3/AP-1a probe. These shifts could be supershifted with antibodies againts c-jun and c-fos, but not ATF-2 (Figure 15B). When cells were stimulated with shear stress and TNF-α simultaneously, the increased binding failed to appear. This suggests that at least the PEA3/AP-1a element might be involved in the regulation provoked by both shear stress and TNF-α.

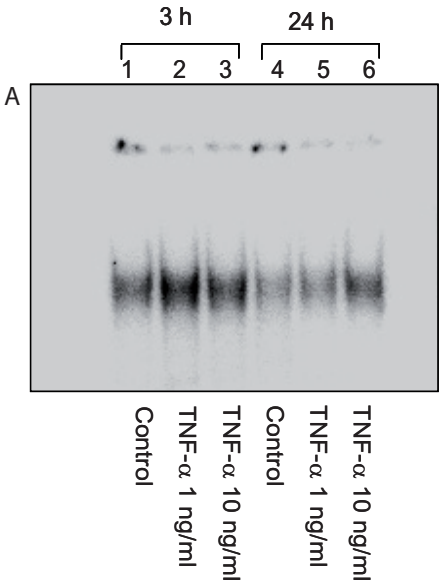


Figure 15A. EMSA showing interactions with the u-PA specific probe containing the AP-1b element. Nuclear extracts from HUVEC exposed to 1 ng/ml and 10 ng/ml TNF-α for 3 and 24h. Data are representative of five independent experiments.

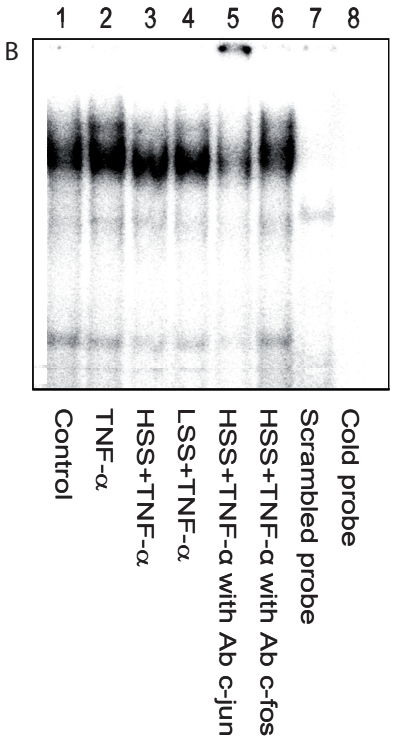


Figure 15B. EMSA showing interactions with the u-PA specific probe containing the PEA3/AP-1a element. Nuclear extracts from HUVECs exposed to TNF-α (1 ng/ml) and high (25 dyn/cm$^{2)}$ or low (1.5 dyn/cm^2) shear stress for 24h. Lane 5-6 with addition of antibodies (Ab) directed against c-jun and c-fos. Lane 7 with a scrambled probe and lane 8 with cold probe to detect non-specific binding. Data are representative of three independent experiments.

DISCUSSION

Differentiated global gene expression response patterns to shear stress and pressure stimulation

Microarray technology was used to obtain global gene expression analysis of shear stress and intraluminal pressure exposed endothelium. To our knowledge, this is the only study in which intact living blood vessels exposed to biomechanical stimulation have been analyzed with microarray technology. However, a few microarray studies on cultured sheared endothelial cells have been reported [136-142]. A large number of genes were found to be responsive to shear stress and intraluminal pressure, which indicates that biomechanical forces are important regulators of cellular functions in the vascular endothelium.

Furthermore, the responses to elevated shear stress or intraluminal pressure were highly differentiated, and the majority of genes (87%) only responded to one of the two factors, which suggests that shear stress and pressure partly activate distinct signaling pathways. This hypothesis is supported by studies of MAP kinase activation in endothelial cells exposed to shear stress and cyclic strain [143]. Using traditional methods of mRNA analysis of single genes, shear stress and cyclic strain have been found to change the expression of some genes in opposite directions. For example, endothelin-1 expression has been observed to be induced by cyclic strain and suppressed by shear stress [144-146]. These observations have led to the hypothesis that the two main hemodymanic forces may have contrasting effects on the endothelium [147]. However, among the 245 genes regulated by both forces in my experiments, only 35 genes were regulated in the opposite direction.

To validate the microarray results, we selected seven genes involved in hemostasis for further analysis with real-time RT-PCR. Overall, the two quantification methods showed a similar response pattern. To further analyze the difference between the two expression patterns emerging from the stimulated endothelium, responsive genes were classified into functional groups using the Proteome database [131]. Again, the response patterns broken down into functional groups were clearly distinct for the two stimuli. A striking difference among the regulated genes was that increased shear stress in contrast to pressure stimulation mainly down-regulated endothelial genes. This is in agreement with previous *in vitro* studies in which laminar shear stress has been shown to have a "quiescent effect" on many genes [137, 138]. From a more general point of view, these findings suggest that the endothelial cells of the vessel wall have the capacity to discriminate the type of deformation force imposed by blood flow and pressure.

Methodological issues in microarray-based gene expression profiling

Although the microarray technology is a powerful tool in global gene expression analysis, criticism regarding its specificity and reproducibility has been raised. In particular, problems relating to normalization, probe annotation, and subtraction of the mismatch probe signal have been discussed.

Pre-processing of raw data is performed by the use of an algorithm that includes background correction, normalization and summarization methods. Recent studies have shown that the results on gene expression data from microarray analysis to a large extent depend on the algorithm used. When gene expressions from the same data set were calculated with different algorithms, varying degrees of overlap of the regulated genes was observed with the different methods [148, 149]. Consequently, the choice of pre-processing method has a major impact on differential expression analysis [150, 151], but they are continuously improving. Comparisons of different platforms have shown both limited concordance of the results [152-154], and lately, high agreement between different platforms [155, 156]. The reason for improved agreement might be due to more developed statistical methods [149]. When comparing the Affymetrix and the Applied Biosystems platforms, Bosotti *et al.* found an 80% overlap of regulated genes between the two platforms [156].

Each transcript on the GeneChip is represented by several probes. Condensing the information from the different probes to produce a single number that best captures the expression level of a particular RNA transcript is a critical step in processing the microarray data [150]. Large variation in intensity among the probes for each transcript is common and might be due to alternative splicing, GC content, and cross-hybridization. Therefore, simple summary statistics such as mean and median may not be sufficient to reflect the full information of the expression levels. For that reason, the use of weighted probes has been suggested [157, 158].

Furthermore, errors in the probe sequence on the Affymetrix arrays have been reported [159, 160]. For the HG U133A arrays used in our experiments, 12% of the probes were reported to be non-specific, which could lead to cross-hybridization of multiple genes and give false positive signals. Also, 8% of the probes were reported to be mistargeted due to sequence errors and since they would not match any transcript they will produce false negative signals [159]. Consequently, a set of probes for a certain transcript might measure more than one transcript [161, 162]. For each Affymetrix probe pair, a mismatch probe with the middle residue changed to the complementary base is included, which is an attempt to adjust for non-specific binding, but it has now been established that the mechanism for cross-hybridization is different for mismatch probes than for perfectly matched [163]. This might explain the fact that in a substantial fraction of probe sets (30%), the mismatch intensity is actually higher than the perfect match probe intensity [164]. Owing to some of these uncertainties of the microarray technology, selected genes of interest require confirmatory tests with real-time RT-PCR for validation. Despite this, I believe that this powerful technique, when used with modern bioinformatics, and with knowledge of its limitations, has the potential to generate new hypothesis and to elucidate how biomechanical forces regulate groups of proatherogenic and antiatherogenic genes in the vessel wall.

Normalization for real-time RT-PCR analysis of biomechanically stimulated endothelium

Real-time RT-PCR is one of the most common methods for confirmation of data from microarray analysis. However, due to its sensitivity, this method may introduce other

sources of variability. A key step to reduce this imprecision is to carefully consider the process of data normalization. Normalization is usually performed by comparing expression of target genes to the expression levels of some endogenous reference genes that are assumed to be constitutively expressed. However, this requires in turn that the reference genes must be evaluated and demonstrated to not be affected by the experimental condition [165]. In Study II, ten different presumable reference genes were analyzed from the microarray data. Five of these genes were further assayed by real-time RT-PCR analysis in order to evaluate their suitability as reference genes of biomechanically stimulated endothelium.

In general, five major sources of variability may introduce error variation in mRNA quantification; biological variability, variations in yield and purity of the extracted RNA, efficiency of the reverse transcription step, variations in the PCR amplification conditions, and imprecision in the detection of amplicon amounts. The findings of this work show that the variabilities associated with PCR amplification conditions, as well as imprecision in the detection of amplicon amount, are very small with the TaqMan method. All five genes had tube-to-tube coeffients of variation, which illustrates the combined variability of amplification and detection errors, below 1%. Thus, the major part of the analytical impression apparently is introduced by pre-PCR preparatory procedures or, alternatively, by the biological variability. Normalization with a reference gene can minimize differences in steps prior to PCR amplification, such as RNA yield, accuracy of quantification, and the efficiency of the cDNA synthesis. Thus, use of an endogenous reference gene has been shown to control for most of the variability associated with preparatory procedures [125].

Reference genes suitable in biomechanical studies of endothelium

As demonstrated by the present findings, four of the five reference genes were not affected by shear stress stimulation. The TfR gene showed a very high coefficient of variation in the real-time RT-PCR analysis and the mRNA level was down-regulated in the microarray results, making it unsuitable as a reference gene. In the pressure stimulation experiments, TfR was the only gene affected by the experimental condition. Although average expression levels were mainly unaffected by mechanical stimulation, there was a considerable variability of the mRNA levels in individual segments exposed to high and low stimulation conditions when analyzed by real time RT-PCR. Since this individual variability may introduce a substantial random error in the gene expression determination, not only average, but also individual variability should be considered when selecting reference genes. Overall, the variabilities were somewhat lower in the pressure than the shear stress series.

To accurately compare expression levels of reference genes, they need normalization to compensate for pre-PCR variation. In order to address this circular problem, between gene correlations of the individual ΔC_T high-low differences were calculated. For some of the reference genes, a consistent pattern was found. Thus, if a large positive ΔC_T high-low was observed for one gene in a particular vessel pair, a large difference in the same direction was found also for the other genes. This finding suggests that systematic variations in the pre-PCR preparatory procedures were the

main sources of variability rather than the specific effects of the experimental situation. For other genes, in particular HPRT and TfR, these correlations were lower and much more variable, which indicated that they are less stable in their expression levels. To circumvent the problem of evaluating reference genes without normalizing them, microarray data, which is normalized without reference genes, can also be used [166-169].

Normalization could be performed either with the composite mean expression value of multiple reference genes, or by comparison with a single reference gene. Vandesompele *et al.*, suggest the use of the geometric mean of the expression to identify appropriate housekeeping genes [170]. With multiple references, the contribution of each reference is smaller and the possibility of artifactual changes due to potential experimental influence of one reference gene is reduced [169].

On the other hand, the use of multiple reference genes is more labour-intensive and expensive. Furthermore, provided that a carefully validation of the current experimental condition has been performed, it appears that both single and multiple reference gene approaches can be used to obtain reliable gene expression data when using the TaqMan method.

Smooth muscle cells in intact vessels respond to shear stress

The expression of P2 receptors in shear stress stimulated endothelial cells and smooth muscle cells was evaluated in study III. Surprisingly, no response of any of the analyzed receptors was observed in endothelial cells, whereas in the smooth muscle cells the expression of the $P2X_1$ receptor was suppressed and the $P2Y_2$ and $P2Y_6$ receptors were induced. Most previous studies have focused on the response of the endothelial cells to shear stress, since the endothelium is believed to be the main sensor of the shearing force. However, it has been suggested that a shear stimulus may also affect the vascular smooth muscle cell layer directly or indirectly.

A direct effect of shear stress has been suggested to be mediated by the transmural pressure gradient, which drives transmural interstitial flow through the fenestral pores in the internal elastic lamina [29, 30]. Calculations imply that the proximal layers of smooth muscle cells experience 10 - 100 times higher shear stress than more distantly located cells [171]. Indeed, this distribution of induced P2 receptors was observed in our experiments when protein expression patterns were analyzed with immunostaining (Figure 9). However an alternative and possibly more likely explanation for this observation is that the SMCs located immediately beneath the endothelium are affected indirectly by shear stress through some kind of chemical signal transduction from the endothelial cells.

$P2X_1$, the most abundantly expressed P2 receptor in SMCs [66] and the receptor primarily coupled to vasoconstriction [48], was suppressed by high compared to low shear stress. A down-regulation of the contractile capacity of the SMCs would, in turn, lead to a reduced vascular tone. This mechanism may contribute to the vascular wall relaxation frequently observed in response to shear stress.

Both $P2Y_2$ and $P2Y_6$ receptors were induced by high shear stress in SMCs. These receptors are mitogenic in their phenotype, and might play a more important role in SMC growth, migration, and differentiation [77, 172]. Extracellular nucleotides have the capacity to stimulate DNA synthesis and protein synthesis and the effect is mediated by activation of P2Y receptors, most clearly shown for $P2Y_2$ and $P2Y_6$ receptors [75, 172]. The pattern of the altered expression of $P2X_1$, $P2Y_2$, and $P2Y_6$ in SMCs may indicate that shear stress changes the phenotype from a contractile to a mitogenic state, which might promote vascular remodeling as an adjustment to a flow-induced increase of the lumen diameter.

Regulation of vascular tone is an important function controlled by biomechanical forces. Extracellular nucleotides are involved in this process by binding to P2 receptors and thereby mediating both vasoconstriction and vasodilation. Vasoconstriction is mediated by ATP release from the sympathetic nerves in the adventitia [173]. Shear-induced ATP release from the endothelium mediates release of NO, EDHF, and prostaglandins, that further augment relaxation of the SMCs [57, 59, 60]. The production of NO is induced by an increase in intracellular Ca^{2+}, which has been shown to be mediated by the $P2X_4$ receptor [67]. Experiments with $P2X_4$-deficient mice suggest an important role for this receptor in mediating vasodilation induced by shear stress [69]. This effect might contribute to the regulation of the $P2X_1$, $P2Y_2$, and $P2Y_6$ receptors in SMCs in our experiments.

The results of the present work cannot exclude the possibility that P2 receptors in the endothelium are regulated by shear stress, since the duration of the experiments was short (6h) and only endothelium of umbilical origin was studied. The regulation of P2 receptors in shear stressed endothelium and smooth muscle cells has not been fully explored and only one previous study reports on regulation of P2 receptors in response to shear stress. In this study, $P2X_4$ gene expression was suppressed by shear stress in HUVECs after exposure for up to 24h. The effect was reported to be mediated by binding of Sp1 [174].

Protective effect of shear stress on u-PA expression in endothelial cells exposed to inflammatory stress

The effect of shear stress on gene expression in endothelial cells has been intensively studied, but less is known about the interplay between shear stress and inflammation. These previous studies have mainly included vascular adhesion molecules [175-177]. We therefore wanted to investigate the interplay between these factors on u-PA expression. Cultured HUVECs were exposed to shear stress, TNF-α, or a combination of both. Shear stress alone dramatically suppressed u-PA expression compared to control conditions, whereas TNF-α up-regulated the expression of u-PA. When combining shear stress and inflammatory stimulation, we found that TNF-α induced u-PA expression was reduced by more than 50%. Thus, the main finding of Study IV is that shear stress exerts a strong protective effect on the TNF-α induced u-PA expression in endothelial cells. Expression of u-PA has previously been reported to be differentially expressed by turbulent and laminar shear stress in human coronary artery endothelial cells [82] Proinflammatory cytokines, such as TNF-α and IL-1β are known to induce

u-PA expression in the endothelium [97, 107]. u-PA activity and u-PA dependent matrix degradation is also increased by TNF-α [107]. However, to our knowledge, no previous study has investigated the interplay between shear and inflammatory stress on u-PA expression.

The endothelium of normal, non-inflamed vessels shows negative or only weak staining for u-PA, whereas immunostaining of inflamed endothelium has been shown to be increased [104]. This points towards that endothelial u-PA preferentially has a role when the vessel wall is exposed to inflammatory stress. Thus, the impact of the regulated response to shear stress might be higher when the endothelium is simultaneously exposed to inflammation.

Interestingly, van Hinsbergh *et al.* reported that TNF-α increased production of u-PA was found only at the basolateral side of the cells [107], which indicates that endothelial derived u-PA acts preferentially on extracellular matrix and smooth muscle cells, rather than being secreted endoluminally into the circulation. Indeed, studies show that induced u-PA expression can stimulate the migration and proliferation of smooth muscle cells [99] and mediate arterial neointima formation [100]. Expression of u-PA is increased in atherosclerotic human aortas, carotid arteries [85, 94, 98] and u-PA overexpressed in the arterial wall caused acute vascular constriction and accelerated atherosclerotic lesion formation [101]. This stresses the importance of the u-PA system as a possible key enzyme in the initial process of atherosclerosis. It also implicates that shear stress regulation could be of major importance particularly in the presence of inflammation.

Interplay between shear stress and inflammation

In contrast to the opposing effects of inflammatory and shear stress stimulation on u-PA, the expression of PAI-1 was induced by both stimuli. Furthermore, the increase was additive when cells were simultaneously exposed to shear stress and TNF-α. Previously, PAI-1 has been reported to be both induced [178] and repressed [179] by shear stress. Furthermore, in contrast to our results, PAI-1 expression has been reported to be regulated in opposite direction in response to shear- and TNF-α- stimulation [179]. This discrepancy may be due to methodological differences in the use of shear device and quantification methods.

The importance of PAI-1 as an inhibitor of the fibrinolysis has been discussed mostly in association with t-PA in the circulation. However, there is still no convincing evidence that circulating PAI-1 origins from the vascular endothelium [180]. On the contrary, the majority of PAI-1 from cultured human endothelial cells has been reported to be located beneath the cells in the extracellular matrix [181], which indicates that endothelial PAI-1 might be more important in regulating basolateral fibrinolysis. Taken together with the finding that TNF-α induced endothelial u-PA was confined to the basolateral side of the cells [107], this may indicate that endothelial-derived u-PA and PAI-1 require a fine-tuning to regulate interstitial fibrinolysis.

VCAM-1 and eNOS are well-documented shear and TNF-α responsive genes, and the interplay between these stimuli has previously been reported [175, 176, 182, 183]. We analyzed and confirmed the expected counteracting effect of shear stress on TNF-α mediated VCAM-1 induction and eNOS reduction, respectively. This again, suggests that shear stress may act protectively against moderate inflammatory stimulation. These results also validate our model and experimental set-up as the findings are well in accordance with findings obtained in other model systems.

u-PA binds to its receptor, u-PAR, to increase its activity, and we therefore wanted to investigate the influence of shear stress on the receptor. However, no major response to shear stress was observed on u-PAR mRNA expression, which indicates that u-PAR may play a minor role in shear stress regulated pericellular fibrinolysis. Further, the mitogenic effect of u-PA has been shown to be independently of u-PAR [99], and the kinetic advantage of u-PAR-bound u-PA in plasmin generation is reported to be less significant for processes requiring days or weeks, such as arteriogenesis and possibly atherosclerosis [184].

TNF-α mediated induction by JNK activation

Experiments with pharmacological inhibitors specific for the signaling pathways activated by TNF-α, showed that the TNF-α induced u-PA expression was at least partly blocked by the JNK pathway inhibitor. The down-stream effector of this pathway is the AP-1 superfamily, which among other factors include c-jun, c-fos, and ATF-2. There are two AP-1 elements in the u-PA enhancer that have the capacity to bind these transcription factors. Therefore, to search for potential mediators of the cytokine effect we performed gel shift experiments. These studies showed an increased binding of TNF-α treated nuclear extract to both AP-1 sites, and could be confirmed with supershift experiments when using antibodies against c-jun and c-fos, but not ATF-2. When stimulating cells with both TNF-α and shear stress simultaneously, the increased interaction between nuclear extracts and the AP-1b probe observed with TNF-α alone, failed to appear. Thus, activation of the JNK pathway could at least partly explain the result on u-PA transcript levels. However, further experiments are needed to explore this mechanism.

CONCLUDING REMARKS

In the present work we have used an *ex vivo* whole-vessel perfusion model that allowed us to study the complex effects of hemodynamic forces on the endothelium and underlying smooth muscle cells. By experimentally segregating the different biomechanical forces imposed on the vessel wall and analyzing the effect by broad gene-array technique, we were able to explore how the endothelium responds to each stimulus separately but in the presence of a physiological level of the other. Interestingly, the two main forces shear and tensile stress, induced by the blood flow and pressure, were found to induce distinct global gene expression responses. This indicates that the endothelial cell has a well-developed ability to distinguish between these forces and thereby respond in a highly differentiated manner to each. Previously, our knowledge of cellular functions of endothelial cells has mainly been based on studies of isolated cultured endothelial cells. The advantage of such systems includes the possibility to use advanced molecular biological analysis. However, our *ex vivo* perfusion model facilitates studies on cellular and sub-cellular mechanisms in the perspective of whole-vessel physiology with preserved interaction between the different cell populations.

Most previous studies of biomechanical forces have focused on the effect of shear stress, since it has been shown to be of major importance both in vascular physiology and pathophysiology. *In vivo*, the level of shear stress is maintained constant during alterations in blood flow by rapid adjustments of the vessel lumen. This feedback system allows acute regulation in order to accurately control blood flow supply and the efficiency is further potentiated by a local regulatory mechanism, since each cell is equipped with the ability to sense the shearing force. This feedback system is particularly important in meeting altered metabolic demands. In this work, we have shown that besides the impact on the endothelium, shear stress also affects the smooth muscle cells in vessels with intact endothelium. P2 receptors in smooth muscle cells, which are important in regulating vascular tone and remodeling, promptly responded to increased shear stress by changing the expression profile from a contractile to a mitogenic phenotype. This further stresses the importance of studying the endothelium in physiological relevant models with intact vessels that permit the physiologic cross-talk between different cell types.

Recently, inflammation in the vascular wall has emerged as an important determinator of vascular dysfunction and progression of atherosclerosis. It has been suggested that shear stress has the capacity to protect the endothelium from some of the negative effects of inflammatory stress. In the present studies we found that shear stress was able to strongly interfere with the inflammation response in a protective way. This observation further illustrates the potency of shear stress stimulation on endothelial cells.

Taken together, the present studies support the view that biomechanical forces acting on the vessel wall are important stimuli that communicate information to the endothelium and smooth muscle cells, which in turn is crucial for their role as key controllers of vascular wall functions. Increased knowledge of how each of the two main mechanical factors exerts its effects may help in understanding how the combined biomechanical setting could play a role in the pathophysiological mechanisms behind clinically important conditions such as hypertension and atherosclerosis.

CONCLUSIONS

Biomechanical forces regulate a large number of genes in endothelial cells. Shear stress and intraluminal pressure induce different gene expression response patterns.

The endogenous control genes; GAPDH, β-actin, and CYC can be used as reference genes in real time RT-PCR gene expression studies of biomechanically stimulated endothelium.

Shear stress decreases the expression of the contractile $P2X_1$ receptor and increases the expression of the mitogenic $P2Y_2$ and $P2Y_6$ receptors in smooth muscle cells.

Shear stress protects against inflammatory stress-induced u-PA expression, whereas PAI-1 expression is additively induced by shear stress and inflammation.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Hjärt-kärlsjukdomar är den vanligaste dödsorsaken i västvärlden. Hjärtinfarkt och slaganfall uppkommer vanligen på grund av bristningar i åderförkalkade områden i blodkärlen, vilket får till följd att en blodpropp täpper till blodflödet. Blodkärlen består av endotelceller som är det innersta lagret mot blodbanan och av lager av muskelceller och stödjevävnad. Åderförkalkade områden uppträder under endotelcellerna, företrädelsevis i förgreningar av blodkärlen och beror bland annat på den lokalt rådande biomekaniska miljön. De biomekaniska krafterna styr flera av blodkärlens viktiga funktioner t ex regleringen av kärldiametern och strukturella förändringar av kärlväggen. Blodkärlen utsätts kontinuerligt för biomekaniska krafter via blodflödet och blodtrycket. De två huvudsakliga krafterna är shear stress som är friktionskraften som blodflödet utövar på kärlväggen samt tryckkraften, den spänningskraft i kärlväggen som genereras av blodtrycket. Syftet med den här avhandlingen var att undersöka hur de biomekaniska krafterna påverkar blodkärlen.

För att studera de biomekaniska krafterna användes ett datorstyrt kärlperfusionssystem som är utvecklat på vårt laboratorium. Hela levande blodkärl i navelsträngar utsattes för olika nivåer av shear stress och tryck genom att reglera flödet och trycket i systemet.

I delarbete I ville vi undersöka om endotelcellerna kan känna skillnad på tryck och shear stress. Detta undersöktes genom att endotelceller i blodkärlen som exponerats i perfusionssystemet, isolerades och analyserades med microarrayteknik. Med microarrayteknik kan genuttryck av över 20 000 gener mätas samtidigt. Resultaten visade att ett stort antal gener regleras i endotelcellerna och att många av dessa gener svarade olika beroende på vilken nivå av biomekanisk kraft de utsattes för. Dessutom var det endast ett litet antal gener som reagerade på båda typerna av krafter. Eftersom mönstren av genuttryck var olika för de två krafterna verkar det som endotelcellerna kan känna skillnad på tryck och shear stress.

I delarbete II analyserades tio kontrollgener. Vid studier av genuttryck är det viktigt att använda kontrollgener för normalisering som inte påverkas av den experimentella behandlingen. Därför utvärderades vilka av de vanligaste kontrollgenerna som är lämpliga att använda när man undersöker hur gener i endotelcellerna påverkas av biomekaniska krafter. Det visade sig att några av de analyserade generna påverkades av behandlingen medan minst tre av generna är lämpliga att använda.

I delarbete III studerades hur P2 receptorer påverkas av shear stress. P2 receptorer är mottagare av en viss typ av signalmolekyler (t ex ATP) som styr många viktiga biologiska funktioner. De förmedlar bland annat kärlens sammandragande effekt men även strukturella förändringar av kärlväggen. Både endotelceller och muskelceller från blodkärl utsatta för shear stress i kärlperfusionssystemet analyserades. Det visade sig att inga av det undersöka P2 receptorerna i endotelcellerna hade påverkats medan genuttrycket och proteinnivåer av $P2X_1$ hade minskat och $P2Y_2$ och $P2Y_6$ i glatta muskelceller hade ökat. Detta i sin tur kan delvis förklara hur kärlet anpassar sig till flödesförhållanden.

I delarbete IV ville vi undersökta interaktionen mellan inflammation och shear stress eftersom även inflammation är en viktig faktor vid uppkomsten av åderförkalkning. Detta studerades i en shear stress stimuleringsmodell där odlade endotelceller från navelsträngskärl utsattes för olika nivåer av shear stress. Cellerna utsattes även för inflammationsstimulering genom att den inflammatoriska molekylen TNF-α tillsattes till systemet. Eftersom enzymet urokinas-plasminogenaktivator (u-PA) har visat sig vara viktig för uppkomsten av åderförkalkning ville vi studera hur det påverkades av shear stress med och utan samtidig inflammationsstimulering. Även u-PAs hämmare plasminogenaktivatorinhibitor-1 (PAI-1) undersöktes. Resultaten visade att shear stress minskade genuttrycket av u-PA medan det ökade kraftigt av inflammation. Samtidig stimulering med shear stress och TNF-α resulterade i att effekten av inflammation på u-PA näst intill uteblev. Det betyder att shear stress tycks ha en skyddande effekt mot inflammation. PAI-1 inducerades både av shear stress och av TNF-α och kombinationen av båda stimuleringarna resulterade i en additiv effekt.

Sammanfattningsvis talar resultaten i denna avhandling för att biomekaniska krafter påverkar uttrycket av ett stort antal gener i kärlväggen och att endotelcellen verkar kunna särskilja mellan shear stress och tryck. Vi har även visat att shear stress kan påverka muskelcellerna i kärlväggen via en viss typ av receptorer och att shear stress har en skyddande effekt mot inflammationsstimulering. Genom att fortsätta att studera hur de biomekaniska krafterna påverkar och förändrar kärlväggen kan man förhoppningsvis öka kunskapen kring varför förändringar i blodkärlen uppkommer. Det skulle också kunna bidra till en större förståelse för sjukdomstillstånd som högt blodtryck och åderförkalkning.

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