ENDOTHELIAL CELL RESPONSES TO THROMBIN AND SHEAR STRESS

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A thesis submitted to the University of London
for the degree of Doctor of Philosophy

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December 2007
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ENDOTHELIAL CELL RESPONSES TO THROMBIN AND SHEAR STRESS

Endothelial cells alter their morphology in response to a combination of chemical and mechanical signals. Both thrombin and shear stress have been reported to activate integrin receptors, calcium channels and downstream Rho GTPases. The aim of the investigation was to contribute in the understanding of the mechanisms involved in the transduction of these signals. Biochemistry and microscopy-based techniques were employed to study the contribution of focal adhesion proteins (FAK, Paxillin, Src).

Both thrombin and shear stress altered the phosphorylation status of FAK, paxillin and Src and the response was more rapid under shear stress conditions. The patterns of phosphorylation followed by specific tyrosine residues were similar for the two responses, but shear stress responses were cell type and magnitude-dependent. Despite similarities in focal adhesion protein phosphorylation patterns, changes in cell morphology differed between thrombin- and shear stress- treated endothelial cells. It was found that, thrombin-induced morphological changes preceded its effects on the phosphorylation of FAK, Src and paxillin while the shear-stress-induced morphological adaptations followed changes in the phosphorylation status of these proteins.

Thrombin induced increased activity of RhoA, not Rac1, but, only in the shear stress response, was stress fibre formation associated with increased phosphorylation of myosin light chain protein. These results suggest that the two stimuli signal through distinct pathways leading to different morphological changes.

The role of the calcium-dependent proteases calpain-1 and calpain-2 was investigated using calpain inhibitors and isoform specific siRNAs. It was found that calpains and specifically calpain-2 plays an important role in the regulation of focal adhesion turnover. Calpain-2 could thereby play a central role in both the thrombin and shear stress responses in endothelial cells.
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Abbreviations

AcTub  Acetylated tubulin
ADP   Adenosine diphosphate
ALLN  Ac-Leu-Leu-Nle-H, aldehydes aka calpain inhibitor-I
ANOVA Analysis of variance
AP    Alkaline Phosphatase
APS   Ammonium persulphate
ATP   Adenosine triphosphate
BAEC  Bovine aortic endothelial cells
BCIP  5-bromo-4-chloro-3-indoyl phosphatase
BSA   Bovine serum albumin
C-terminus Carboxyl terminus
CaMK  Calcium calmodulin-dependent protein kinase
CALP  Calpeptin
CALP1  Calpain-1 or μ-calpain
CALP2  Calpain-2 or m-calpain
CI-I  Calpain inhibitor-I
DMEM  Dulbecco's modified eagle medium
DTT   Dithithreitol
ECL   Enhanced chemiluminescence
ECM   Extracellular matrix
EDTA  Ethylene-diamine-tetra acetic acid
ERK   Extracellular signal-regulated kinase
cNOS  Endothelial nitric oxide synthase
FAK   Focal adhesion kinase
FITC  Fluorescein isothiocynate
HUVEC Human umbilical vein endothelial cells
G    Globular
GAP  GTPase activating protein
GDI   Guanine nucleotide dissociation inhibitor
GEF   Guanine nucleotide exchange factor
GPCR  G-protein coupled receptor
GST   Glutathione S-transferase
GTP   Guanine triphosphate
HRP   Horse radish peroxidase
FCS   Foetal calf Serum
IP$_3$ Inositol (1, 4, 5) trisphosphate
IPTG Isopropyl β-D-thiogalactoside
MLC   Myosin light chain
MLCK  Myosin light chain kinase
MLCP  Myosin light chain phosphatase
MT    Microtubule
MTOC  Microtubule organizing centre
N-terminus Amino terminus
NBT   Nitro blue tetrazodium
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>NP40</td>
<td>Nonidet P40</td>
</tr>
<tr>
<td>PAEC</td>
<td>Porcine aortic endothelial cells</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet endothelial cell adhesion molecule-1</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol biphosphate</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethysulphonyl fluoride</td>
</tr>
<tr>
<td>PAR</td>
<td>Protease activated receptor</td>
</tr>
<tr>
<td>pY, pS, pT</td>
<td>Phospho-tyrosine, phospho-serine, phospho-threonine</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated coiled-coil kinase</td>
</tr>
<tr>
<td>RheB</td>
<td>Rho extraction buffer</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation buffer</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine isothiocyanate</td>
</tr>
<tr>
<td>TXA2</td>
<td>Transcellular thromboxane</td>
</tr>
<tr>
<td>VE</td>
<td>Vascular endothelial</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial cell growth factor receptor</td>
</tr>
<tr>
<td>WAVE</td>
<td>WASp-like verproline homologous protein</td>
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ACKNOWLEDGEMENTS

I was always encouraged to do the degree of Doctor of Philosophy. I had my reservations both before and during its progress. Nearing the end, I would like to thank everyone who at some point or another has contributed in the encouragement from the bottom of my heart; it turned out to be a valuable experience from which I have learned a lot both about the field of science and about myself.

First and foremost I would like to thank my supervisor Professor Anne Ridley, for all she has done throughout these four years. Special thanks go to all members of the CCB laboratory. For reasons familiar to them, I would like to thank, Drs Jaime Milan, Eva Carnuda Morollon, Aleks Ivetic, Kirsi Riento and Ritu Garg, as well as Richard Foxon. Special thanks go to a recent addition to the group Dr. Ferran Valderrama for the final push and motivation, to Dr. Kostas Lykostratis for his shear stress support as well as to Dr. Nicholas Raymond, Dr. Francisco Vega and Sandra Kuemper for providing me both with help and entertainment during my stay at the CCB laboratory. Last but definitely not least. I would like to thank Parag Bhavsar and Undine Gottesbuehren for reasons I can not begin to describe.

My thanks are not enough to express my gratitude and love for my family and especially my parents, without whom I would not have been able to do any of the things I have done so far, and in the absence of whom nothing I will do in the future will bare the same impact as in their presence. The same but to a bit lesser degree is true for Zacharias Koullouros, Elena Vassiliou, Stelios Vorkas, Kostas Kozakis, Katerina Papaphilotheou and Chryssie Jacovides who have stood by me when they did not have to and hope they will continue to do so for much longer. A final thank to Dr. Leonidas Philaktou, who even though may not be aware of it, has helped me to begin and continue my career in science.

Finally, I would like to make sure to point out that for the people I have failed to mention I will express my gratitude to personally as this page is not long enough to refer to everyone I am lucky enough to have me in their lives.
1 INTRODUCTION

1.1 The endothelium
Endothelial cells form the inner lining of blood vessels and provide a biological barrier between circulating blood in the lumen and the rest of the vessel wall (Hofer et al., 1999). The vascular endothelium is not just a passive barrier but plays an active role in controlling fluid and nutrient transport between the blood and the vessel wall. Along with permeability it locally controls vascular tone and growth. The endothelial monolayer is involved in many physiological processes including angiogenesis as well as the defensive processes of blood clotting and inflammation. Endothelial cells also divide and migrate during wound healing. In addition to physiological processes, many pathological processes involve the disruption of the endothelium e.g. atherosclerosis (Cines et al., 1998), demonstrating the importance of maintaining a functional endothelium.

In vivo, endothelial cells are exposed to a combination of mechanical and chemical stimuli, including growth factors such as vascular endothelial growth factor (VEGF), cytokines such as thrombin, and mechanical stress such as cyclical stress or shear stress (Ballermann, 1998). The endothelium responds to these signals in part by altering its morphology (Hofer et al., 1999).

Together with the environment, endothelial responses depend on their genetically “programmed” function determined by their developmental origins (Stevens et al., 2001). Embryological studies suggest that the endothelium arises either from microvasculatory segments (vasculogenesis) or macrovascular segments (angiogenesis) (Stevens et al., 2001). Thus, endothelial cells differ between and within organs both in their structure and function (Aurrand-Lions et al., 2001).

1.2 Intermolecular junctions
Electron microscopy studies have revealed heterogeneity in the structure of junctional complexes in endothelial cells (Aurand-Lion et al., 2001). In vertebrates there are four types of junctions enabling attachments between adjacent cells: tight junctions, adherens
Introduction

junctio ns (zonula adherence), gap junctions and desmosomes. The presence of junctional heterogeneity suggests that tissue-specialization of blood vessels may be dependent upon the molecular architecture of intermolecular junctions.

**Figure 1.1: Cell junctions**

Diagrammatic representations of cell junctions: structures especially abundant in epithelial and endothelial tissues. Junctions consist of protein complexes allowing adhesions between neighbouring cells, important for functional integrity and regulated permeability of the tissues. Left: diagram illustrating and describing tight junctions (in epithelial membranes and parts of the vasculature), gap junctions (also found in endothelial cells) and desmosomes (Taken from Marieb, 2004). Junctional proteins, participate in the formation of junctions in a tissue specific manner. Endothelial specific junctional proteins include Claudin-5 and VE-cadherin (Aurand-Lion et al., 2001). Right: diagram illustrating adherence junctions in endothelial cells which includes the endothelial specific VE-cadherin (Taken from Bezzoni and Dejana, 2004).

1.3 The cadherin family of junctional proteins: VE-cadherin

Cadherins are transmembrane homophillic adhesion proteins that link to catenins
providing anchorage of adherens junctions to the cytoskeleton (figure 1.1). The assembly and disassembly of the dynamic cadherin/catenin complex can be regulated by cytokines and growth factors (Petzelbaue et al., 2000). Endothelial cells also express other cadherins, including N-cadherin (not in adherens junctions) and P-cadherin (in trace amounts), which are also expressed in other cell types, but VE-cadherin is specific for endothelial cells (Liaw et al., 1990; Lampugnani et al., 1992; Breier et al., 1996). It is specifically located at the lateral cell surface and is important in controlling the permeability properties of the endothelium (Lampugnani et al., 1992).

Inhibition of VE-cadherin via antibodies has been shown to increase permeability and neutrophil transmigration in vivo (Gotsch et al., 1997). Thrombin or VEGF stimulation of endothelial cells results in the disassembly of adherens junctions, re-distribution of VE-cadherin and a consequent increase in endothelial permeability (Lampugnani et al., 1992; Seebach et al., 2005). Increasing permeability also occurs in the absence of calcium, as cadherins require the binding of calcium for the formation of adherens junctions; in the presence of calcium chelators, BAPTA and EGTA, endothelial cell contraction and disappearance of the junctional protein from intracellular adhesions occurs (Lampugnani et al., 1992).

VE-cadherin null mice are unable to from vascular structures (Carmeliet et al, 1999). VE-cadherin at cell-cell adhesions mediates growth stop at confluence. Neither vessel formation nor growth inhibition can be mediated by PECAM-1 in the absence of VE-cadherin (for references see Petzelbaue et al., 2000). PECAM-1 (platelet endothelial cell adhesion molecule-1) also binds to catenins modulating their localization and sequestration thereby modulating adherence junction dynamics (Biswas et al., 2005). Separate from its function in cell adhesion, immunoprecipitation studies suggested that VE-cadherin is involved in the transduction of mechanical signals by acting as an adaptor protein linking shear stress activated PI3K to vascular endothelial growth factor receptor-2 (VEGFR2) (Tzima et al., 2005; Fleming et al., 2005).

VE-cadherin appears to be specifically important for vessel formation, growth inhibition and the inhibition of endothelial permeability in a calcium-dependent manner.
1.4 Extracellular signals: shear stress and thrombin

Morphological adaptation to external signalling is a process associated with extensive remodeling of the cytoskeleton and sites of adhesions. As is the case with all cells, morphological changes in response to extracellular signals require that the signal is sensed by receptors on the cell surface and transduced from the outside to the intracellular environment.

1.4.1 Shear Stress

Shear stress is the mechanical frictional force exerted on endothelial cells as a result of blood flow, which together with cyclical strain (wall tension exerted in three dimensions during systole and diastole) constitute the hemodynamic forces influencing the behaviour of vessels (Lehoux et al., 2006). As blood is pumped from the heart through the circulatory system it flows in large arteries into the smaller arterioles, capillaries, vanules and back to the heart via veins (Figure 1.2). The magnitude of shear stress differs depending on vessel diameter, the speed of blood flow and the viscosity of blood (Figure 1.3). Under physiological conditions, changes in blood flow are responsible for acute regulation of vessel tone, the development and growth of blood vessel structure during embryogenesis, and the chronic remodelling of adult blood vessels following changes in the mechanical environment (Resnick et al., 2003).

![Image taken from Marieb, 2004](image已久的)
Introduction

Endothelial cell

Intimal Layer

Fibroblast Normal Veins

1  1  Normal ArteriesMedial Layer Adventitial Layer

Figure 1.3: Shear stress

a) The structure of blood vessels showing haemodynamic forces exerted on the endothelial monolayer. Figure kindly donated by Dr. Kostas Lykostratis b) Shear stress equation and its parameters: \( \tau_s = \frac{\mu Q}{\Pi R^3} \)

Shear stress magnitude (dyn/cm²)

- Normal Veins
- Normal Arteries

Figure 1.3: Shear stress

a) The structure of blood vessels showing haemodynamic forces exerted on the endothelial monolayer. Figure kindly donated by Dr. Kostas Lykostratis b) Shear stress equation and its parameters: \( \tau_s = \frac{\mu Q}{\Pi R^3} \), \( \mu = \) blood viscosity (N-s/m²), \( Q = \) velocity (m/s), \( R = \) radius. Shear stress force is usually expressed in dyn/cm²; 1 dyn/cm² = 0.1 N/m²
c) Shear stress magnitudes associated with specific vessels: healthy blood vessels usually experience low shear stress, which is not harmful to endothelial cells. Between arteries and veins, arteries experience high and veins experience low shear stress. The differences in shear stress force between vessels can be attributed to vessel diameter.

In addition to various shear stress magnitudes there are also different types of shear stress associated with the different vessels, Turbulent flow occurs at arterial bifurcations where blood of high shear stress is forced to change directions. Laminar flow occurs in vessels with a diameter less than 0.5 mm thick where the flow is unidirectional and uniform in velocity (Lelkes et al., 2003). The role of shear stress in vascular pathology is not very well understood, yet both the type and magnitude of shear stress appear to be implicated in the localized development of the cardiovascular disease atherosclerosis. While some regions of the arterial tree, in particular those associated with constant laminar blood
flow, never succumb to atherosclerosis, others such as the arterial bifurcations are highly susceptible (Davies et al., 2007). The response of the endothelium therefore differs depending on the type of flow and to a certain degree the magnitude of flow to which they are subjected.

Endothelial cells adapt to shear stress by changing their morphology in order to reduce the mechanical load exerted on them. Shear stress induces changes in gene expression, cell shape, differentiation, proliferation and cell cycle progression. It is possible that different types and magnitudes of shear stress activate independent signalling pathways exerting diverse effects on the endothelial monolayer. The processes of transduction of mechanical signals (mechano-transduction) requires that the detection of the signal takes place via receptors expressed on the cell membrane also known as mechanosensors or mechanotransducers. The exact molecular basis of mechanotransduction in endothelial cells has yet to be determined but among the most studied are cell substratum adhesions (see Chapter 1.6), which are believed to act as mechanosensors regulating signal transduction cascades. In addition to focal adhesions where mechanosensing takes place via integrin receptors (see Chapter 1.5), shear stress is able to directly activate the adhesion molecule PECAM-1 (Tzima et al., 2005; Fleming et al., 2005). Shear stress has also been shown to activate other receptors such as calcium channels (see Chapter 1.9) and vascular endothelial growth factor receptor-2 (VEGFR-2) (Wang et al., 2002). Furthermore, VE-cadherin has been implicated in contributing to mechanosensing via the formation of a VE-cadherin-β-catenin-VEGFR-2 complex (Shay-Salit e al., 2002).

1.4.2 Thrombin
Thrombin is a multifunctional serine protease whose activity is induced upon cell wounding through proteolytic conversion of its inactive form prothrombin into its active form thrombin. It provides a signal for wound healing and inflammation by catalyzing the conversion of fibrinogen into insoluble fibrin, a process critical for blood coagulation (Shapiro, 2003; Furie and Furie, 2003). In addition, thrombin has cytokine and growth-factor functions which include cell proliferation and chemotaxis in cell lines, and endothelial cells, and modulates endothelial cell permeability (Zhou, 1998).
Endothelial exposure to thrombin occurs when blood vessels are injured. Wound formation leads to the recruitment of platelets in the subendothelium and their consequent exposure to tissue factor (Ofosu, 2002). Tissue factor (thromboplastin) is a protein present in subendothelial tissues and ultimately leads to the formation of thrombin (Figure 1.4).

![Coagulation Cascade Diagram](image)

**Figure 1.4: The coagulation cascade**

Cytokines released from monocytes induce the expression of tissue factor on endothelial cells which via the interaction with factor VIIa initiate a cascade leading to the production of thrombin. Once released in the blood stream thrombin is able to act on the endothelium. The diagram has been modified from Ofosu, 2002.

While the procoagulant process is mediated by an array of at least six plasma proteins (prothrombin, factor VII, factor IX, factor X, factor V and factor VIII) and one tissue protein tissue factor (TF), the anticoagulant process is governed by four plasma proteins, antithrombin III (AT-III), protein C, proein S, the tissue factor pathway inhibitor (TFI) and by one membrane-bound protein thrombodulin (Butenas et al., 1999). From empirirical and theoretical models of the coagulation system it became clear that the
process of blood coagulation system behaves in such a way that synergy between procoagulants and inhibitors (anticoagulants) determine responses to stimuli and that the procoagulant initiating stimulus must be at a certain level to bring about uncompromised generation of thrombin (Butenas et al., 1999). Experiments carried out by Butenas et al. (1999) reported that out of the aforementioned 12 proteins, which are explicitly associated with the maintenance of blood fluidity and protection from vascular injury, prothrombin and AT-III are the proteins whose concentration range affects the amount of total thrombin available as well as the maximum levels and rates of generation. The amount of thrombin generated in the procoagulant response is time-dependent and reaches a maximum of 1500 nM within 5 min of the propagation phase (figure 1.5).

Figure 1.5:
Thrombin generation in the “synthetic plasma” mixture
Thrombin generation initiated with 1.25 pM tissue factor to reaction mixture containing mean plasma concentrations of procoagulant proteins in the presence of phospholipids. Data taken from Butenas, 2002.

Thrombin exerts most of its effects via the activation of G protein-coupled-receptors of the PAR family. Protease-activated receptors (PARs) are part of the superfamily of related G protein-coupled receptors (GPCRs) comprising of four family members PAR₁, PAR₂, PAR₃ and PAR₄. PARs are highly expressed in platelets and are detectable in a variety of tissues and cells including the endothelial cells (Steinhoff et al., 2005). By cleaving the N-terminal extracellular domain of PARs, serine proteases enable the recruitment of Gi/o, Gq, and G12/13 protein families to the receptor resulting in a physiological response (Vanhauwe et al., 2002). Amongst the serine proteases are thrombin and trypsin which activate PAR₁, PAR₄ and PAR₂ respectively. PAR₃ appears to function as a co-factor for PAR₄ rather than induce signal transduction, nevertheless
Introduction
differential expression of PAR3 and PAR4 in different tissues suggests that PAR3 has an
independent role that needs to be determined (Hollenberg et al., 2003). PAR1 constitutes
the best studied receptor for thrombin, and its cloning led to the discovery of the other
family members (Hollenber et al., 2003). Integrin receptors are also activated in response
to thrombin possibly via a process that involves cross talk between the two receptor
families, integrins and PARs (Juliano, 2002).

1.4.3 Thrombin and shear stress: biological significance through vascular pathology
Links between shear stress and thrombin have already been established. Shear stress has
been reported to activate G-proteins, also involved in the activation of integrins and
PAR1 receptors, by increasing fluidity of the lipid bilayer (Haidekker et al., 2000). It has
been shown that, in the absence of GPCRs, G proteins reconstituted in liposomes are
capable of increasing their activity when exposed to shear stress (Gudi et al., 1996). In
addition, cells exposed to different levels of shear stress were observed to down-regulate
the expression of PAR1 by affecting the rate of the PAR1 transcription (Nguyen, 2001).
Although there are some reservations as to the physiological significance of thrombin
signaling in endothelial cells, it has been proposed that thrombin like shear stress plays a
key role in the development of atherosclerosis, which is one of the leading causes of
mortality worldwide. Ofosu (2002) reports that failure to localize and adequately regulate
thrombin production can have pathological consequences including the development and
propagation of atherosclerosis and tumour development.

The word atherosclerosis is derived from the combination of two Greek words, athero
and sclerosis, translated into paste and hardness respectively. Atherosclerosis involves
accumulation of intracellular and extracellular lipids, monocytes and T-lymphocytes, in
the innermost layer of blood vessels (Libby, 1993). The build up of fatty plaques on the
inside of vessel walls gives rise to what is known as an atherotic plaque or atheroma,
whose continued growth results in the narrowing of the lumen, reduced blood flow, the
formation of blood clots and finally blocking of the lumen, heart attack and stroke
(Feldman et al., 2002). One of the initial conditions contributing to the development of
atherosclerotic plaques is the formation of intercellular gaps associated with endothelial
Introduction

cell contraction and reorganization of filamentous actin (F-actin). Development of atherosclerosis has therefore also been associated with changes in the structure and function of cell-cell junctional proteins (Simionescu et al., 2007; Weinberg, 2004).

Accumulating evidence recognizes the endothelial monolayer as critical in the initiation, development and possible management of atherosclerosis (Healy and Quinlan, 2004). There is a correlation between locations of atherosclerotic plaques and the haemodynamic forces exerted on the endothelium due to blood flow (Davies et al., 1995); plaques tend to develop at areas exposed to low magnitude turbulent/oscillatory shear stress whereas regions associated with constant laminar blood flow appear to have atheroprotective properties.

As with shear stress, the role and significance of thrombin in vascular pathology are not very well understood. In vivo experiments have linked short-term dietary-mediated regression of atherosclerosis with enhanced anticoagulant responses to thrombin (Lentz et al., 2002). Thrombin stimulation of endothelial cells enhances the activity of arginase, an enzyme highly active in atherosclerotic aortas (Ming et al., 2004). There is compelling evidence implicating PAR1 in mediating thrombin-induced vascular permeability and subsequent oedema suggesting that PAR1 antagonism could be used in the treatment of inflammatory diseases (Major et al., 2003). Based on feature similarities between chronic inflammation and atherosclerosis it has been proposed that thrombin plays a key role in the development of atherosclerosis, both by promoting fibrin deposition into the atherosclerotic vessel wall and also by signalling through PARs (Nicholson and Hajjar, 1998; Major et al., 2003).

1.5 Integrins

Integrins constitute a highly conserved family of transmembrane linkers between intracellular and extracellular proteins that have a dual function; they function both primarily as “adhesive structures” but are also important as “signalling molecules” (Faull and Ginsberg, 1996). They are involved in the transduction of signals from the extracellular domain to the cytoplasm, a process termed “outside-in signalling” as well as from the inside of the cell to the outside, known as “inside-out signalling”, both of which
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are of equal importance to the function of the receptors in mediating morphological changes.

Integrins are non-covalently associated heterodimeric cell surface adhesion molecules (Giancotti, 2003) consisting of two subunits; the α chain (120-180 kD) and β chain (90-110 kD), each of which crosses the cell membrane once (Faull and Ginsberg, 1996). In vertebrates there are 18 α and 8 β subunits, the combination of which gives rise to 24 known αβ pairs (Luo et al., 2007). The parts of the α and β chains that protrude from the cell collaborate to form the receptor for extracellular binding partners (Horwitz, 1997).

Prior to ligand binding, integrins are found in a closed-headpiece-bent state, in which ligand affinity is weak (Faull and Ginsberg, 1996). Once bound, the ligand induces a series of conformational changes via which integrins achieve an open-headpiece-extended conformation, increasing the receptor's affinity for the ligand and hence the ligand-integrin interaction. The change from low to high affinity integrin conformations requires the movement of several integrin domains formed via interactions of the α and β chains.

With the use of total internal reflection microscopy to help the visualization of FITC-labelled anti-β1 integrin antibody in endothelial cells, it was observed that integrin binding to the extracellular matrix is followed by the clustering of integrins supplied from the intracellular space allowing for more efficient interactions and increased binding between the cells and the extracellular matrix (Kawakami et al., 2001).

Activated integrins have been shown to localize at focal adhesions (van der Flier and Sonnenberg, 2001). Their cytoplasmic tails are very small in size (30 to 50 amino acids), do not contain catalytic or consensus protein-protein interaction motifs (van der Flier and Sonnenberg, 2001) but are responsible for the targeting of the receptor at these sites; studies using deletions, mutations and chimeric constructs revealed that the β subunit is responsible for the targeting of integrins (Hayashi et al., 1990) but that localization at focal adhesions is inhibited by the α subunit. Once ligand is bound, the inhibition is
relieved and activated integrins are able to translocate to focal adhesions (LaFlamme et al., 1992).

Exactly how integrins are activated by thrombin or shear stress is not yet fully understood, but integrin activation does occur in response to both stimuli. Activation of αVβ3 integrin by shear stress has been confirmed with the use of ligand-mimetics that exhibit high affinity for the receptor (Pampori et al., 1999). Blocking of integrin-binding sites for extracellular ligands prevents the shear stress induced mechanotransduction via the integrins, indicating the requirement for a dynamic interaction of integrins with extracellular matrix (ECM) ligands for the process (Jalali et al., 2001). Following shear stress application, matrix-specific ligation of integrins occurs resulting in the initiation of signalling cascades (Tzima et al., 2001).

For thrombin, the mechanism of integrin activation is even less straightforward; a series of studies revealed a strong association of a protein (integrin-associated-protein; IAP) not only with the cytoplasmic tails of β3 integrins but also with heterotrimeric G proteins (Brown and Frazier, 1999), suggesting the indirect activation of integrins by thrombin via activation of G-proteins. There are also indications that integrin activation could be occurring via the action of Rap, which has been shown to activate integrins (Bos, 2005).
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1.6 Sites of cell adhesion as sites of signal transduction: functional overview of focal adhesion sites and their components

Several different adhesion receptors are involved in mediating cell interaction with the extracellular matrix and with neighbouring cells, offering structural support to the cell and the tissue as a whole. Cell-cell interactions are mediated via four types of intermolecular junctions discussed in Chapters 1.2 and 1.3. The sites at which integrin receptors are clustered and adhere to the extracellular matrix are termed focal contacts. Focal contacts are very small in size but mature into larger complexes known as focal adhesions.

Focal adhesions are sites via which cells attach to the underlying substrate, at positions where the plasma membrane is within 15 nm of the extracellular surface (Zaidel-Bar et al., 2004). In addition to integrins focal adhesions contain a large number of structural and cytoskeletal molecules, some of which are also signalling proteins (Figure 1.5) (Burridge et al., 2006). Reflection contrast microscopy and electron microscopy were used to identify focal adhesions in fibroblasts, a spear tip-like structure up to 10 μm long and 0.5 μm wide (Abercrombie, 1971 cited in Jockusch et al., 1995). Components of focal adhesions include some proteins with the ability to bind cytoskeletal components, providing integrins with a link to the cytoskeleton (Figure 1.5).

Upon integrin activation and clustering, focal adhesion proteins are recruited to integrins, and some exhibit changes in their phosphorylation status at serine, threonine and tyrosine residues of which tyrosine are the most studied.

Together, protein recruitment and changes in phosphorylation status facilitate the transduction of the integrin-sensed signal regulating cytoskeletal rearrangements and inducing morphological changes. The sequence via which the different proteins are recruited or phosphorylated in response to stimulation is not easy to determine. Yet advances in experimental procedures have contributed to our understanding, of the roles played by certain focal adhesion proteins in integrin-induced signal transduction. Some of these proteins are talin, focal adhesion kinase (FAK), Src and paxillin.
Introduction

Figure 1.6: Focal adhesions to focal adhesions: Simplified diagram showing focal adhesions which includes the cytoplasmic tail of integrins and a few focal adhesion proteins that will be discussed later on. Increased recruitment of proteins to focal adhesions in response to stimulation results in the maturation of these sites into focal adhesions. Focal adhesions: highly organized yet dynamic aggregates of more than 100 different proteins, suggesting a considerable functional diversity (Zamir and Geiger, 2001).

1.6.2 Talin
Talins are large mutli-domain proteins with a globular head and a rod like tail, which are regulated by calpain and by binding to multiple molecules (figure 1.6). The result of talin proteolysis by calpain is the separation of the amino- head domain (47 kDa) from the carboxyl-tail (220 kDa sometimes reported as 190 kDa) both of which localize to focal adhesions (Nuckolls et al., 1990) (see Chapter 1.10.1).

There exist two very similar talin proteins in mammals, namely talin 1 and talin 2, of which the former has been studied more extensively. Talin 1 is thought to be responsible for exerting a small mechanical force on the extracellular matrix at the leading edge of migrating cells (Jiang et al., 2003). In this study, it was hypothesized that talin was the first protein to bind the integrin-fibronectin complex exerting the force necessary for the formation of cell protrusions until other proteins are recruited in response to stimulation. The presence of talin alone though, appears not to be enough for triggering the assembly of the focal adhesion protein complex; chimeric integrins derived from constitutively active receptor tyrosine kinases that were proven to constitutively send integrin signals (Martin-Bermudo and Brown, 1999 cited in Tanentzapf et al., 2006) were able to sequester talin but very limiting amounts of other cytoplasmic proteins (Tanentzapf et al., 2006).
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The importance of the scaffolding function of talin has been demonstrated though studies that reveal the detachment of the integrins from the actin cytoskeleton in the absence of the talin (Tanentzapf et al., 2006). Of equal importance to talin function and the integrin-cytoskeleton interaction is its involvement in integrin clustering; mutations that inhibit talin interaction with integrins, result in the inhibition of integrin clustering (Tanentzapf et al., 2006).

The importance of the talin function in endothelial cells subjected to shear stress was demonstrated via talin overexpression resulting in relieving the integrin suppression imposed by integrin blocking antibodies, upon exposure of bovine aortic endothelial cells to 12 dyn/cm2 of laminar shear stress (Orr et al., 2006). In addition to proteolysis, talin undergoes phosphorylation by serine, threonine as well as tyrosine kinases (Ratnikov et al., 2005), about which little is known. Studies carried out in endothelial cells indicate that talin exhibits a degree of tyrosine phosphorylation under basal conditions, which nevertheless remains unchanged in response to thrombin stimulation (Schaphorst et al., 1997).

1.6.3 FAK

Focal adhesion kinase (FAK) is a 125-kDa protein tyrosine kinase involved in signal transduction pathways and a close relative to Pyk2. FAK and Pyk2 are cytoplasmic tyrosine kinases and can be activated by a variety of stimuli (Zheng, 1998). FAK has several tyrosine residues whose phosphorylation regulate its FAK activity and function (Figure 1.7). Deregulation of FAK has been implicated with the development of cancer (Miyazaki et al., 2003).

Both thrombin and shear stress activate FAK in endothelial cells. While comparisons between published data reveal the latter to be more rapid, the exact timing of phosphorylation as well as whether phosphorylation actually occurs appears to be cell type specific (Holinstat et al., 2006; Shikata et al., 2003; van Nieuw Amerongen et al., 2004; Shikata et al., 2003; Li et al., 2002; Zaidel-Bar et al. 2005).
1.6.4 Src
Src belongs to a family of tyrosine kinases comprising of nine closely related proteins (Frame et al., 2004). They are membrane-associated proteins which upon recruitment to focal adhesions are responsible for the phosphorylation of several proteins (Figure 1.8).

Both thrombin and shear stress have been reported to induce the activity of Src in endothelial cells evident by increases in pY416-Src levels very early in the time-course (Bijli et al., 2007; Okuda et al., 1999). In shear stress, calcium is required for Src activation; calcium chelators, Src family-specific kinase inhibitors (PP1) and kinase inactive Src inhibit shear stress induced phosphorylation of p130Cas and actin filament assembly (Okuda et al., 1999).

1.6.5 Paxillin
Paxillin is a 68-kDa protein which as mentioned above participates in complexes formed at focal adhesions upon integrin activation. The protein has an important function as a scaffold facilitating interactions between other proteins of the complex (Figure 1.9). Close relatives to paxillin containing LD motifs are Hic5, leupaxin and PaxB; alignment of the LD motifs revealed high conservation between the proteins and exhibited binding to FAK and vinculin both of which contain highly conserved paxillin binding sudomain (PBD).

Localized regulation of the phosphorylation of Y118-paxillin, has been reported in PAECs under shear stress, at the upstream edge relative to the direction of flow. Shear stress induced cellular movement (sub-confluent cultures or wound healing) requires rearrangement of the actin cytoskeleton involving two opposing processes at the two extremes of the cell. The involvement of paxillin in locally regulating cytoskeletal dynamics could constitute an important mechanism for the realization of the two distinct effects.
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Calpain-mediated proteolysis:

Interacts strongly with F-actin

Allows talin to act as a scaffold connecting ECM with the cytoskeleton.

Active role in nucleation of actin filaments, capping and cross-linking

(Niggli et al., 1994).

<table>
<thead>
<tr>
<th>Interacts with integrins and phospholipids</th>
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<tr>
<td>N-terminus 47 kDa and Globular Head 190 kDa Rod</td>
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<td>High in helical content and rich in alanine repeats</td>
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| Interaction of praline-rich motifs resulting in antiparall talin homodimer |
| Layillin binding domain |
| PIP-2-binding sites |
| FAK-binding domain |

| Figure 1.7: The talin molecule |

Diagrammatic representation of the talin molecule structure and binding partners with annotated functions and posttranslational modifications. Integrin interaction: the N-terminal ‘head’ of talin binds to the cytoplasmic β subunit of the integrin tail. Phospholipid interactions: The specific association of of N-terminal ‘head’ of talin with phosphatidylinositol 4,5-biphosphate (PI4,5P2) induces conformational changes in the talin molecule, which results in the exposure of an integrin binding site allowing for the targeting of talin to integrins (Martel et al., 2001).
Autophosphorylation site: Upon stimulation, either by β1/β3 integrins or by regulatory peptides and lipids acting on G-protein coupled receptors FAK autophosphorylates and becomes activated. It is then able to bind Src (Short et al., 1998). Y397 Also provides docking sites for p120RasGAP.

Src-phosphorylated: Y576 Y577
Further stimulates kinase activity
(Cornillon et al., 2003)

Src-phosphorylated: Y861 Y925
Provide docking sites for Grb2 (growth factor receptor binding protein 2) and 190RhoGEF (see Chapter 1.7)

Proline rich motifs: bind to SH3 domain of adaptor protein p130Cas (Crk associated tyrosine kinase substrate) and GRAF (Rho GTPase GAP) (see Chapter 1.7).

FAT domain
Comprises focal adhesion targeting sequence via which FAK is recruited to focal adhesions (Schaller et al., 1993). Also contains PBS (paxillin binding site) and talin binding sites

FERM domain via which FAK binds to integrins

Kinase domain via which FAK is able to phosphorylate other focal adhesions

Figure 1.8: The FAK molecule
Diagrammatic representation of FAK structure. Binding partners and the main tyrosine phosphorylation sites are indicated. Once autophosphorylated, FAK leads to phosphorylation of other focal adhesion proteins via its catalytic central domain, contributing to signal propagation (Cornillon, 2003). FAK can also contribute to the phosphorylation of downstream proteins indirectly via the recruitment of other kinases such as Src to focal adhesions.
Residue resides in Src activation loop. When phosphorylated (intrinsically) Src becomes activated. The conformation change induced by Y527 de-phosphorylation and Y416 phosphorylation displacement of the pY416 from the binding pocket occurs allowing the substrates to gain access Y416 to the kinase domain (see Pertone and Sap, 2000).

When phosphorylated Src is found in a closed conformation mediated by binding of SH2 with pY527. Its de-phosphorylation via phosphatases e.g. SHP1 leads to conformational changes and Src activation. Mutation results in Src overactivity and development of cancer. (Fincham et al., 2000)

| - Linker |
| SH3 domain: recognizes proline-rich sequences enabling Src to bind to other focal adhesion |
| SH2 domain: recognizes phosphotyrosine residues enabling Src to bind to other focal adhesion proteins eg FAK. FAK binding also induces protein domain displacement, resulting in Src adopting its open active conformation. Also binds to paxillin. |
| Kinsase domain: responsible for phosphorylation of other focal adhesion proteins in its vicinity |

**Figure 1.9: The Src molecule**

Targeting of Src to focal adhesions via its protein-protein interactions results in the phosphorylation of other focal adhesion proteins such as paxillin, to which Src binds via its SH2 and SH3 domains (Brown et al., 2005; Weng et al., 1993; Sabe et al., 1994; Turner and Miller et al., 1994).
Phosphorylated by FAK or Src and possibly via other kinases independently of FAK-Src complex such as Brk (Chen et al., 2004; Guo et al., 2006; Ishibe et al., 2003; Plopper and Ingber, 1993)

Phosphorylation occurs after its recruitment to the FAK-Src complex formed as a result of integrin activation (Plopper and Ingber, 1993).

Figure 1.10: The paxillin molecule
Simplified diagrammatic representation of paxillin domains. The best studied tyrosine sites of phosphorylation and binding partners are shown. Via its direct interaction with vinculin, paxillin is able to associate with the actin cytoskeleton, while additional LD motifs link the protein directly to the cytoskeleton through interactions with tubulin (for cytoskeleton see Chapter 1.3). Via its binding to p120RasGAP pY31/118-paxillin is involved in the regulation of the cytoskeleton (see Chapter 1.7).
1.6.6 Localized phosphorylation of focal adhesion proteins

Signalling systems often involve a cascade mechanism of sequential phosphorylation of proteins, in this case focal adhesion proteins. Although several focal adhesion proteins are reported to be phosphorylated in focal adhesions, it is not yet clear whether the phosphorylation takes place prior to the translocation of the protein to the sites of adhesion or vice versa. In fact, there is evidence that different focal adhesion proteins become phosphorylated at different cell locations. For example, with the use of phospho-specific and total paxillin antibodies, Ballestrem et al. (2006) showed that paxillin phosphorylation occurs after its translocation to focal adhesions, while by comparing the localization of paxillin and FAK fused to cyan or yellow fluorescent protein (CPP or XPP) in live NIH 3T3 fibroblasts, the group suggested that FAK phosphorylation is broadly distributed throughout the cell (Ballestrem et al., 2006).

1.6.7 Dynamic nature of focal adhesions

An important feature of focal adhesions is their dynamic nature which enables the assembly and disassembly of these structures at localized cell areas was required (Zamir and Geiger, 2001). With the exception of talin and vinculin binding to each other, interactions between focal adhesion proteins are of low affinities (Burridge and Mangeat, 1984). Although this report only deals with a limited sample of focal adhesion proteins it suggests that by requiring the binding of many proteins with low affinities, the complex not only becomes highly versatile but also gains speed in its assembly as well as disassembly (Jockusch et al., 2007).

1.7 Rho GTPases

Members of the small Rho GTPase (Ras-homologous proteins, ~25 kDa) family are the key mediators of many signalling pathways and regulate cytoskeletal organization and cell adhesion. 22 members have been identified in mammals, making up the Rho, Rac, Cdc42, Rnd, RhoBTB and RhoT subfamilies (Bustelo et al., 2007). Out of these, RhoA, Rac1 and Cdc42 are the best characterized (Ridley, 2000). When activated, Rho GTPases modulate the activity of effectors via which they are able to regulate cytoskeletal reorganization and cell adhesion. Expression levels of Rho GTPases can be regulated at
Introduction

the transcriptional level, yet the induction of their activity is dependent upon post-translational modifications and protein-protein interactions (Figure 1.10).

RhoA, Rac1 and Cdc42 have been implicated in endothelial cell responses to shear stress (Li et al., 1999; Tzima et al., 2001) and thrombin (Birukova et al., 2003), associated with remodelling of the cytoskeleton, cell-cell adhesion, focal contact formation and maturation into focal adhesions.

Known RhoA regulators connected with focal adhesions include p190RhoGAP, which in endothelial cells is phosphorylated and thereby activated by Src (Chang et al., 1995; Burridge et al., 2006), p190RhoGEF a binding partner of FAK (Figure 1.7) and p190RhoGAP which when in complex with p120RasGAP is inactive. Paxillin and FAK are binding partners for p120RasGAP (Figures 1.7 and 1.9) and compete with p190RhoGAP for their binding to the protein. Once paxillin/FAK bind p120RasGAP, p190RhoGAP is free from the complex and able to inactivate RhoA. The localized suppression of RhoA activity by pY31/118-paxillin was observed to occur in cell adhesion and migration (Tsubouchi, 2002).

Shear stress has been shown to cause a rapid yet transient increase in the activation of RhoA in HUVECs which after returning to basal levels was followed by an increase in Rac1 and Cdc42 (Wojciak-Stothard and Ridley, 2003). In subconfluent HUVECs, the increase in RhoA activity correlated with endothelial cell contraction, while the activation of Rac1 and Cdc42 coincided with shear stress-induced re-spreading of cells and their elongation in the direction of flow. RhoA inhibition and Rac1 activation also appear to be a requirement for cell alignment of bovine aortic endothelial cells exposed to higher magnitudes of flow (Tzima et al., 2001). Even though shear stress responses in the two cell types are mediated by the action of Rho GTPases the relative importance of each Rho GTPase is dependent upon cell-type, confluency and possibly magnitude of shear stress.
Requires post-translational addition of a lipid group (geranyl-geranyl or farnesyl) dictated by the CAAX box sequence, found in the carboxyl-terminus of the protein. Cleavage of the AAX moiety then enables the carboxymethylation of the carboxyl-terminal cysteine (Takai et al., 2001) and localization of the complex to the membrane.

**Figure 1.11: Regulation of Rho GTPases**

With the exception of Rnd family members, Rho GTPases exist in two conformations: a GTP-bound (active) form and a GDP-bound (inactive) form. Rho-GDP has a low affinity for Rho effectors and the conformational change induced by the binding of GTP increases the affinity (Van Aelst and D'souza-Shorey, 1997). The ratio between the two conformations is regulated through the action of GAPs and GEFs. Rho GTPases interact with many effectors and specificity is achieved via the ability of effector proteins to recognize distinct regions within the effector domain of the Rho proteins, as well as certain sequences outside the effector region (Bishop and Hall, 2000).
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In endothelial cells there is evidence for a crosstalk between Rho GTPases suggesting mutually exclusive phenotypes generated by Rho and Rac, including transient inactivation of Rho by active Rac and Rac associated phenotypes induced by Rho inhibition (cited in Civelekoglu-Scholey et al., 2005). While both RhoA and Rac1 have been shown to be involved in the formation of integrin-based adhesions, their contributions are different. Rottner et al., (1999) showed that transfection of NIH 3T3 fibroblasts with constitutively active Rac in the presence of Rho inhibitors results in the formation of focal complexes independent of Rho activity, which serve as precursor sites for maturation into focal adhesions in a Rho-dependent pathway associated with down-regulation of Rac. The mutual exclusiveness between Rho and Rac activities has also been demonstrated for thrombin-induced endothelial cell contraction, which stimulates RhoA activation but no change in Rac activity (van Nieuw Amerongen et al., 2000).

1.8 The cytoskeleton

The cytoskeleton, as the name implies, is the cellular “skeleton” or “scaffolding” of the cell. The higher eukaryotic cytoskeleton is composed of three primary elements, the microfilaments (actin filaments), intermediate filaments and microtubules, which frequently act together in regulating cell shape, organelle motility and maintaining structural integrity (Cooper and Pollard, 1982). The filamentous nature of the cytoskeletal components arises from polymerization of different proteins whose assembly or disassembly is dictated by their intra- as well as extra-cellular environment. The dynamic nature of the cytoskeleton allows for its rapid re-organization.

1.8.1 Actin cytoskeleton

In a confluent endothelial monolayer, the majority of actin is polymerized (~ 70%), and turnover rates of actin filaments are very slow. Upon removal of contact inhibition the cytoplasmic content of actin filaments decreases dramatically correlating with an increase in filament turnover (Dewey, 1984).

Filamentous actin (F-actin) arises from the polymerization of individual actin subunits also known as G-actin (globular actin). Actin polymerization is a reversible process that
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involves the activation of actin monomers, the generation of oligomers via the process of nucleation (catalyzed by actin nucleators such as the Arp2/3 complex), and polymer elongation (Pollard, 2007). ATP-bound actin monomers undergo conformational changes induced by the binding of magnesium (Mg\(^{2+}\)). Following monomer activation is the processes of nucleation (rate-limiting step) that involves the assembly of either two or three actin monomers into an oligomer representing the nucleus for polymerization which leads to the generation of actin filaments (Pollard, 2007). F-actin has a double-helical arrangement of uniformly oriented monomers. De-polymerization of F-actin is induced after a period of growth via hydrolysis of the bound ATP as a means of controlling filament length. ATP hydrolysis results in severing and the de-polymerization of actin filaments by the action of ADF/cofilin. Cofilin interacts with both globular and filamentous ADP-actin (Van Troys and Dewitte, 1997). The action of cofilin allows the dissociation of actin monomers, which can bind to profiling promoting the exchange of ADP to ATP and thereby activating the monomers and allowing for their recycling and assembly into new actin filaments (Cooper and Pollard, 1982). Actin stabilization is achieved via interaction of capping proteins such as CapZ with the "barbed" end of polymers preventing the addition or loss of actin monomers.

The early stages of high shear stress application in pulmonary endothelial cells have been associated with actin nucleation involving activation of Arp2/3 complex (Birukov et al., 2002); shear stress induced the translocation of cortactin, a binding partner and activator of the Arp2/3 complex to the cell cortex suggesting it plays a role in initiating localized actin nucleation. Cortactin translocation to the cortex can be regulated via phosphorylated Src, but this was not the case in the shear stress experiments. Instead, Rac was implicated in cortactin regulation. Activation of the Arp2/3 complex has also been suggested to occur in the early stages of thrombin signalling in endothelial cells. Using magnetic bead microscopy, rapid stiffening of the cell membrane within 5 seconds of thrombin stimulation was observed (Bausch et al., 2001). Although not proven it is hypothesized that in response to stimulation, thrombin induces the activation of the Arp2/3 complex via the regulation of either Rac or Cdc42 GTPases. This does not exclude the possibility that thrombin-induced cell membrane stiffening is the result of other mechanisms of action.
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The primary actin structure in endothelial cells is a three dimensional orthogonal network of actin filaments that interconnects the more basal stress fibres; a specific cytoskeletal organization of actin bundles and networks organized by actin filament cross-linking proteins containing actin-binding domains. Depending on which cross-linking protein is bound, parallel or anti-parallel bundles are formed. The distance between parallel bundles is determined by the size of cross-linking protein which in turn allows for the interaction of actin with other proteins such as myosin, a protein involved in regulating stress fibre formation and their contraction (see Chapter 1.8.2) (Wechezak et al., 1989; Satcher et al., 1997; Sacher et al., 1996).

1.8.2 Myosins and MLC phosphorylation

Myosins are motor proteins that move along actin filaments. Most family members comprise of heavy and light chains forming bipolar complexes. Non-muscle myosin II, exists in a closed/bent conformation. Upon phosphorylation of its regulatory light chain, MLC, the protein adopts an extended conformation, is able to form bipolar complexes and thereby becomes activated.

Myosin light chain (MLC) phosphorylation occurs downstream of Rho GTPases. Although it is not yet clear which Rho GTPase is responsible for MLC phosphorylation, it is known that RhoA activates ROCK (also known as Rho Kinase) which regulates MLC indirectly by inducing the phosphorylation of MLC phosphatase, rendering the enzyme inactive and thereby inhibiting MLC de-phosphorylation (Kimura et al., 1996). ROCK is also able to activate MLC directly through phosphorylation of Ser19-MLC (Totsukawa et al., 2003). MLC phosphorylation and activation is also induced by MLCK (myosin light chain kinase) (Totsukawa et al., 2003). The regulation of MLCK activity is dependent upon the availability of calcium. In the presence of calcium, calmodulin is able to bind MLCK inducing a conformational change that relieves the protein from auto-inhibition (Tang et al., 2004). Phosphorylation of MLCK results in decreased affinity of the protein for calcium/calmodulin thereby rendering it inactive (Nishikawa et al., 1994). MLCK phosphorylates MLC at two residues Ser19, the major site of phosphorylation, and Thr18 whose phosphorylation occurs at a slower rate under conditions of maximum
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stimulation (Burridge and Chrazowska-Wodniak, 1996).

Cell contraction is brought about by the transition of MLC from bent to an open/extended conformation upon activation where Ser19 and Thr18 have individual roles; pSer19-MLC shifts the equilibrium between the two conformations towards the open state promoting the assembly of myosin filaments. Phosphorylation of Thr18 then stabilizes this extended conformation (reviewed in Burridge and Chrazowska, 1996) leading to actomyosin contraction. Microinjection experiments using truncated mutants rendering myosin phosphatase constitutively active or inactive in fibroblasts revealed the necessity and sufficiency of MLC phosphorylation in the assembly of stress fibres and focal adhesions (Totsukawa et al., 2000). ROCK and MLCK have distinct roles in the spatial regulation of MLC phosphorylation for the assembly of stress fibres and focal adhesions: ROCK promotes phosphorylation in the centre of the cell whereas MLCK acts at the cell periphery (Totsukawa et al., 2000).

It has been suggested that thrombin-induced endothelial cell contraction is the result of MLC phosphorylation by MLCK, since inhibition of the enzyme abolishes the response (Moy et al., 1996). Prevention of the shear stress-induced MLC phosphorylation with MLCK inhibitors provides evidence for the necessity of the MLCK pathway in endothelial cells exposed to flow (Watanbe et al., 1998). On the other hand, shear stress appears to induce stiffening of the cytoplasm of adherent cells by enhancing actin assembly and inducing contractility (Lee et al., 2006). Enhanced contractility, the result of an increase in the levels of phospho-MLC, proved to be mediated via the ROCK pathway, as inhibitors of the kinase entirely eliminate the response (Lee et al., 2006). Induction of ROCK activity in bovine aortic endothelial cells exposed to high shear stress has been reported to be rapid and transient (Lin et al., 2003). ROCK also mediates thrombin-induced endothelial cell responses, both regulating MLC phosphorylation (van Nieuw Amerongen et al., 2000) and other responses including ICAM-1 expression and enzymatic activity of arginase (Anwar et al., 2004; Ming et al., 2004).
1.8.3 The microtubule cytoskeleton

Microtubules are hollow cylinders consisting of thirteen protofilaments, the result of the end to end polymerization of α and β tubulin dimers with the α subunit of one tubulin dimer contacting the β subunit of the next. The particular orientation of the tubulin dimers results in exposure of only α subunits at one end (-) and only β subunits at the other end (+) of protofilaments. As the protofilaments arrange parallel to one another, the polarity is maintained. The first step in the generation of microtubules is nucleation which generally occurs at the microtubule organizing centre (MTOC). Both tubulin subunits are GTP-bound, yet the association with α-tubulin is more stable than with β-tubulin. Addition of tubulin dimers to the microtubule requires that β-tubulin is in a GTP-bound state. Hydrolysis of GTP to GDP is induced at the (+) end after assembly and due to differences in kinetics between GTP and GDP-bound tubulin, the (+) end of microtubules is prone to de-polymerization. Disassembly is normally prevented by a cap of GTP-bound tubulin at the + tip of microtubules, but under conditions where GTP hydrolysis becomes faster than tubulin addition, then rapid de-polymerization occurs, also known as catastrophe. This effect can be rescued by the formation of a new cap by the addition of GTP-bound tubulin (reviewed in Inoue and Salmon, 1995).

Dynamic instability is an important feature of the microtubule network, especially in migration, and allows cells to rapidly assemble and disassemble their microtubule networks before stabilizing them in the most suitable position. There is also growing evidence that the microtubule network comprises not only long stationary filaments but also short mobile microtubule filaments. The “cut and run” model suggests that through severing, cells are able to mobilize their microtubules which once re-organized are then able to elongate and loose their motility (reviewed in Baas et al., 2005).

The ends of microtubules often target focal adhesion complexes (Kaverina et al., 1998; Krylyshkina et al., 2003) and may thereby induce their disassembly probably through the action of Rho GTPases (Bhatt et al., 2002). The preferential targeting of focal adhesions by microtubules could be the means by which cells are able to establish polarity. An important determinant of polarized movement is the position of the MTOC (cited in
Tzima et al., 2003). In bovine aortic endothelial cells shear stress activates Cdc42 leading to re-orientation of the MTOC at the downstream side of the nucleus relative to the direction of flow (Tzima et al., 2003). This re-orientation of the MTOC is associated with the polarization of Cdc42. Microtubule destabilizing agents such as nocodazole and inhibitors of the motor protein kinesin have been shown to disrupt the polarized cell shape of migrating fibroblasts (Rodionov et al., 1993).

Polarization of the microtubule system is important because of the action of motor proteins which via their actions are able to regulate the reorganization of cell adhesion complexes (McCue et al., 2006): Kinesins for example are involved in the trafficking of molecules and complexes too large to diffuse to the sites where they are required e.g. for the formation of new adhesion sites. The force of kinesin binding to the microtubule pulls the cargo along while ATP hydrolysis assists in the directing the movement towards the ‘+’ end the filament (Mather et al., 2006). There are also motor proteins which with the help of ATP are able to carry cargo along microtubules in the ‘-’ direction, namely dyneins.

It is likely that the microtubule network contributes to thrombin-induced endothelial permeability, though this is not yet well understood. Thrombin has been reported to induce the phosphorylation of the microtubule binding protein Tau at serine residues hence promoting their disassembly, which is attenuated in the presence of microtubule stabilizing agents such as taxol (Birukova et al., 2004). Microtubule destabilization increases levels of MLC phosphorylation via RhoA activation (Burridge and Charzanowska-Wodnicka, 1996) while stabilization of the network appears to attenuate the thrombin-induced actin re-organization and can be reversed through the expression of active RhoA or ROCK (Birukova et al., 2004). The thrombin-induced microtubule destabilization and actin re-organization are mediated by p115RhoGEF, a specific RhoA activator and downstream effector of G proteins (G12/13) (Birukova et al., 2004).

Experiments carried out in fibroblasts have suggested regulation of MLC phosphorylation by the microtubule network. Destabilization of the microtubules by nocodazole leads to
Introduction

the release of the RhoGEF, GEF-HI (Birkova et al., 2006), and subsequent RhoA activation (Krendel et al., 2002), which in turn could lead to the phosphorylation of MLC.

1.8.4 Intermediate filaments
Intermediate filaments are so named due to their diameter (8 to 10 nm) relative to the thin actin filaments and the bigger microtubules. Presently there are about 50 known human intermediate filament proteins, which are differentially expressed in different tissues including vimentin. Due to their dynamic nature intermediate filaments are prominent in cells that withstand mechanical stress such as the endothelium (Fuchs and Weber, 1994). Quantitative analysis of spatiotemporal patterns of intermediate filaments, visualized by green fluorescent protein tagged vimentin transfection in bovine aortic endothelial cells, revealed their rapid displacement in response to high shear stress application (Helmke et al., 2001). Thrombin has been shown to affect intermediate filaments by inducing rapid phosphorylation of vimentin in cultured endothelial cells (HUVECs), linking thrombin stimulation to the phosphorylation of the cytoskeletal protein (Bormann et al., 1986).

1.9 Calcium signalling in endothelial cells
Under resting conditions, cytosolic calcium is maintained at low levels by active transport mechanisms. Upon stimulation, calcium can be released into the cytoplasm from the extracellular environment across the plasma membrane as well as from intracellular calcium stores such as the mitochondria, endoplasmic reticulum (EF), Golgi, endosomes or calciosomes (Villereal and Palfrey, 1989). The early influx can result in the production of one single peak returning to basal levels or sustained calcium oscillations for longer time-periods (Dull and Davies, 1991; Shen et al., 1992).

Phospholipase C (PLC) is activated in endothelial cells both in response to thrombin (Lum et al., 1993) as well as shear stress (Ishida et al., 1997). PLC is responsible for the production of inositol (1,4,5) trisphosphate (IP$_3$) from PIP$_2$. IP$_3$ binds to receptors on the ER membrane opening Ca$^{2+}$ channels thereby allowing stored Ca$^{2+}$ to be released into the cytosol (Villereal and Palfrey, 1989; Alberts, 1994). Ins2,4,5P$_3$ and Ins1,3,4,5P$_4$ are thought to synergistically regulate calcium influx channels allowing calcium entry to the
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cytosol from the extracellular space (Tran et al., 2000).

Transfections of human mammary epithelial cells (HMECs) with PLC and G-protein mutants demonstrated the need for both proteins in the thrombin-induced calcium release from intracellular stores (Vanhauwe et al., 2002). These mutants also inhibited thrombin-induced stress fibre formation. Thrombin-induced formation of stress fibres therefore, requires the activation of PLC in order for calcium to be released from intracellular stores.

The mechanical force exerted on the endothelial surface due to shear stress, is thought to contribute to the release of calcium into the cytoplasm by activating the plasma associated stretch-activated calcium channels (Kwan et al., 2003). Shear stress may also activate other receptors such as VEGFR, linked to the IP$_3$ signalling pathway (Resnick et al., 2003), thereby increasing the rate of internal Ca$^{2+}$ release (Tseng et al., 1995).

The physical effects of calcium are mediated by calcium-binding proteins acting as third messengers for the initial signal. There are a number of calcium-binding proteins of which the best characterized consist of “E-F hand” motifs that provide pockets for the binding of calcium (Villereal and Palfrey, 1989). Examples of “E-F hand” containing calcium-binding-proteins include calmodulin and calpain. Calmodulin is responsible for modulating the activity of several proteins involved in the regulation of the actin cytoskeleton such as MLC (see Chapter 1.8.2). Calpain on the other hand is thought to be involved in the regulation of focal adhesions (see Chapter 1.10).

1.10 Calpain

Calpains are cytoplasmic cysteine proteases. The family comprises “conventional” and the more recently identified “novel” calpains. The former refers to the first calpains identified, namely calpain 1 and 2. Based on sequence homology, a further 14 genes have been identified to belong to the calpain family in vertebrates. As the identification of these genes is mostly based on sequence homology rather than their function, further characterization of the proteins is required for a better understanding of their nature.
These “novel” calpains include tissue-specific calpains (calpain 3a, calpain 8a and 8 b, calpain 12, calpain 13) and more widely expressed members of the family (calpain 5, calpain 7, calpain 9 and calpain 10) (reviewed in Goll et al., 2003). Members of the calpain family can be alternatively divided into “typical” and “atypical” calpains based on the presence of calmodulin-like domains.

1.10.1 Calpain structure and activation
Both calpain 1 and 2 have a calmodulin-like binding domain and therefore belong to the “typical” calpain subgroup. The term calmodulin-like domain indicates the presence of “E-F” hand sequences which as mentioned above provide binding pockets for calcium (Goll et al., 2003). Calpains are calcium-dependent proteases yet calpain 1 and 2 exhibits different requirements for calcium; for half maximal activity in vitro, calpain 1 requires 3-50 μM of cytosolic calcium while the requirement for calpain 2 ranges between 400 to 800 μM (Cong et al., 1989; Goll et al., 2003; Suzuki, 1991). Based on their μmolar and mmolar calcium requirements calpain 1 and calpain 2 are also referred to as μ- and m-calpains respectively.

Both calpain 1 and 2 are heterodimers each comprising a common small subunit (~30 kDa) and a different large catalytic subunit (~80 kDa). The 80kDa calpain subunit can be divided domains I to IV. Homology of calpain molecules with calmodulin occurs at the carboxyl-terminus of the large subunit, comprising 5 “E-F” hand motifs in total, out of which only 4 are involved in calcium binding. Crystallographic structures have identified the fifth hand as being responsible for the dimerization of the two calpain subunits. In addition to domain IV, “E-F” hands have been identified on domain VI, located at the carboxyl-terminus of the small calpain subunit. Through crystallographic and limited proteolysis experiments the binding of calcium at both of these domains, IV and VI, was revealed (reviewed in Goll et al., 2003). Nevertheless, the 30kDa sub-domain has no role in the function of calpain as a protease and is thought to serve as a chaperone maintaining the conformation of the 80kDa subunit (Pal et al., 2001; Yoshizawa et al., 1995).

X-ray crystallography of calpain 2 revealed that in the absence of calcium, the three
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amino-acids comprising the catalytic site of the molecule (Cys, His and Asn) are separated in space due to conformational restrictions imposed by interactions between domain II and the amino-terminus of domain III. These constraints are released by the binding of calcium ions thereby inducing the activation of calpain (Moldoveanu et al., 2002; Reverter et al., 2001a; Reverter et al., 2001b). After the release of constraints, a second structural change occurs as a consequence of the binding of two calcium ions on the protease domain leading to the rearrangement of the active cleft (Moldoveanu et al., 2002) and the activation of calpain. Still, the conformational changes induced by the binding of these calcium ions, are very small leading to the hypothesis that other domains could contribute in binding calcium inducing further morphological changes in order to initiate the proteolytic activity of calpain (Goll et al., 2003).

Under resting conditions intracellular calcium levels are maintained at low concentrations and so is calpain activity, at least in endothelial cells. Activation of calpain normally occurs only in response to increases in the cytosolic calcium concentration which nevertheless fall within the range of 50-300 nM, much lower than the reported requirements for the activation of calpain 2 (Goll et al., 2003). *In vivo* studies investigating the effect of protein-protein interactions and phospholipid binding to calpains, revealed that cytosolic calcium concentrations required for calpain activation are lower than *in vitro*, thereby allowing activation of calpain at physiological calcium concentrations (Arthur and Crawford, 1996; Melloni et al., 1996; Tompa et al., 2001; Melloni et al., 1998; Michetti et al., 1991).

In response to calcium, calpain is able to translocate from the cytosol to the plasma membrane. In the presence of calcium and phospholipids, calpain molecules undergo autocatalytic hydrolysis resulting in the activation of the two calpain subunits (Tompa et al., 2001). Interestingly, autolysis of calpain appears to lower their calcium requirement for half-maximal proteolytic activity without affecting the specificity of the enzyme (Edmunds et al., 1991). As a result of autocatalytic hydrolysis, the 80 kDa subunit is able dissociate from the plasma membrane (Yoshizawa et al., 1995) and act as a protease for its substrates (Moldoveanu et al., 2002; Tompa et al., 2001).
1.10.2 Calpain inhibitors

Calpastatin is an endogenous calpain inhibitor localized in the cytosol and is able to inhibit both calpains 1 and 2 (Goll et al., 2003). In order for calpastatin to function as an inhibitor, both calpain domains IV and VI are required, indicating that the inhibitor can only act on dimeric calpain (Suzuki et al., 2004). Calpastatin inhibits the protein function by blocking the binding of calpains to the membrane via its two conserved regions A and C (Tompa et al., 2002). In addition, calpastatin binds near the active site of calpain via another conserved region, B, thus reducing the rate of autolysis (Tompa et al., 2002).

The role of calpain in various signalling pathways has been broadly investigated by using peptidyl calpain inhibitors derived from mammalian, plant and synthetic sources (Donkor, 2000). Both calpeptin and calpain inhibitor-I (also known as ALLN), are peptidyl aldehydes derived to improve on the disadvantages of the plant-derived peptidyl inhibitor, leupeptin, in terms of cell permeability as well as its specificity to calpain; leupeptin has also been reported to inhibit plasmin, trypsin, papain and cathepsin B (Donkor, 2000). Generation of calpeptin and calpain inhibitor-I involved N-terminal capping with lipophilic substituents resulting in increased permeability but not selectivity; even though both calpeptin and calpain inhibitor-I do inhibit calpain, they are better inhibitors of other neutral proteases such as cathepsin family proteases (Donkor, 2000).

1.10.3 Calpain protease function and substrates

Calpains recognize bonds between domains, thereby hydrolysing its substrate proteins in a very limited manner (Suzuki et al., 2004). Amongst calpain substrates are several focal adhesion proteins. These include the talin (Franco and Huttenlocher, 2005; Beckerle et al., 1987), the kinases FAK and Src (Cooray et al., 1996; Oda et al., 1993), as well as phosphatases such as PTP1B (Shoenwaeler et al., 1997). Via their action, calpains are able to alter the balance between the activities of tyrosine kinases and phosphatases; the calpain-induced decrease in the levels of tyrosine phosphorylation as a result of calcium ionophore stimulation was demonstrated in platelets (Pain et al., 1999), suggesting that calpain could regulate the turnover of focal adhesions by altering the phosphorylated state.
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of focal adhesion proteins. Calpain is a critical regulator of cell migration, the organization of the actin cytoskeleton and focal adhesions; calpain-null fibroblasts have decreased migration rates and cell detachment and abnormal organization of the actin cytoskeleton, a loss of central stress fibres and fewer yet bigger focal adhesions at the cell periphery (Dourdin et al., 2001).

Formation of strong focal adhesions at the cell periphery occurs in response to calpain inhibition, suggesting that calpains are involved in regulating focal adhesion turnover by cleaving focal adhesion proteins resulting in the disassembly of the complex (Bhatt et al., 2002). This notion was further reinforced by observation that point mutations of the talin molecule, which render it resistant to proteolysis by calpain, had no effect on the localization or the binding properties of talin but persisted for longer periods of time at focal adhesions than the cleavable counterpart (Franco et al., 2004), allowing for the conclusion that calpain mediated proteolysis of talin is a rate limiting step in its disassembly from these sites. Mutation of the proline-rich amino-terminus of FAK, has been shown to block calpain-2 binding to FAK and thereby preventing calpain-mediated proteolysis of FAK which prevents focal adhesion turnover and downstream signalling events, suggesting that by inducing proteolysis calpain is able to regulate the activity of focal adhesion proteins, in this case FAK (Carragher et al., 2003).

In addition to focal adhesion proteins, calpains are thought to mediate cleavage of integrin β3 subunit; experiments carried out in bovine aortic endothelial cells, have shown that integrin-induced Rac or Rho activation required the calpain-dependent formation of integrin clusters, yet after the activation of the small GTPases, additional integrin complexes formed independent of the presence of calpain (Bialkowska et al., 2000). These findings suggest that calpain acts upstream of Rho GTPases regulating their activation status required for focal complex and focal adhesion assembly.

Although there is compelling evidence for the localization of calpain molecules to focal adhesions (Beckerle et al., 1987; Huttenlocher et al., 1997), some groups have reported the absence of calpain from these sites (Bialkowska et al., 2000) suggesting that the
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presence of calpain at focal adhesions could occur only under specific conditions such as the breakdown of the complexes in migratory cells (Huttenlocher et al., 1997).

Calpain molecules have also been implicated in regulating transendothelial migration (TEM) under shear stress conditions (Hussain et al., 2005). Inhibition of TEM by calpain inhibition was brought about through impairing the formation of ICAM-1 rich docking structures of endothelial cells, without affecting the motility of the lymphocyte on the luminal surface of endothelial cells. Activation of calpain in endothelial cells has also been associated with the hypoxia induced decrease in endothelial nitric oxide synthase (eNOS) activity via calpain action on heat shock protein HSP90 (Su et al., 2000).

In endothelial cells calpain-1 is involved in regulating focal complex formation and cell spreading by acting upstream of RhoA and Rac1 regulating cytoskeletal reorganizations (Kulkarni et al., 1999; Bialkowska et al., 2000). Calpain-2, has been shown to catalyze hypoxia-induced destruction of cross-links between the plasma membrane and actin filaments (Aono et al., 2001), to be the dominant protease in transcellular thromboxane (TXA₂)-induced neurovascular endothelial cell death, and reduction in its levels is associated with impaired migration and cell proliferation (Qiu et al., 2006).

Thrombin is able to initiate a process via which talin cleavage occurs; upon platelet stimulation with thrombin, intact talin translocates to the Triton-X-100-insoluble fractin along with calpain-1, which then induces proteolysis of talin (Hayashi et al., 1999). Calpain-1-mediated talin proteolysis also occurs when platelets are exposed to shear stress, a process that proved to be dependent on shear stress magnitude; over 50 dyn/cm² where required for 5 min (Fujitani et al., 1997).
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1.11 Primary cells and immortalized cell lines

A major part of the current knowledge on the functions of endothelial cells has come from in vitro experiments carried out in primary cells whose use has been important in setting up relevant experimental models ranging from inflammation to angiogenesis and atherosclerosis (Unger et al., 2002). Immortalized cell lines, exhibiting endothelial cell characteristics are increasingly being used for in vitro experiments attempting to overcome problems associated with the use of primary cells such as, the time consuming process of isolating the cells, the low yields associated with primary cell isolation, the presence of contaminating cells, the progressive loss of viability, the differences in genetic background of the isolates and the lower cost of obtaining and culturing cell lines (Unger et al., 2002). Nevertheless, despite the advantages associated with the use of immortalized cell lines for in vitro experiments, there are also certain disadvantages that need to be taken into consideration when interpreting results. Amongst the disadvantages is the question as to whether the immortalized cell line has retained all of the characteristics of the primary cells it has been derived from and whether the process of immortalization has contributed in altering any of those characteristics.

The immortalized cell line EA.hy926 is a fusion of HUVECs with the A459 lung carcinoma epithelial cell line and constitutes one of the best characterized endothelial cell lines. Generation of EA.hy926 cells involves the hybridization of isolated primary human umbilical vein endothelial cells (HUVEC) of first passage with Rhodamine 6G-treated A549 cells (Edgell et al., 1983). A549 cells were originally derived from a human lung carcinoma and can be selected against using HAT (hypoxanthine, aminopterin and thymidine). While the purpose of the Rhodamine 6G treatment prior to hybridization was to increase the yield of viable hybrids, the addition of HAT in the medium of the fused culture after the hybridization served for the selection of hybrids (Edgell et al., 1983). Hybrid cells that survived in the selective medium were reported by the group to have more chromosomes than either progenitor cell type.

A comparative study of primary endothelial cells and cell lines for the in vitro expression of endothelial phenotypic markers reported that although EA.hy926 cells retain most
endothelial cell characteristics including constitutive expression of von Willebrand factor and CD31 (platelet endothelial cell adhesion molecule, PECAM-1), expression of both vascular endothelial growth factor receptors Flt-1 and KDR and the cytokine-induced expression of the adhesion molecule ICAM-1, they do not retain other endothelial cell characteristics such as the upregulation of adhesion molecules VCAM-1 and E-selectin in response to cytokine stimulation (Unger et al., 2002). The difference in the responses of EA.hy926 cells and HUVECs has also been demonstrated with the use of microarrays in another comparative study (Boerma et al., 2006). In addition to characteristics shared between the two cell types, Boerma et al. (2006) reported the expression of a larger number of genes in EA.hy926 cells than in HUVECs, many of which related to cell cycle control and apoptosis. The group therefore concluded that even though EA.hy926 cells retain most of endothelial cell characteristics it is necessary to carefully select and validate changes in genes that are the focus of studies when using the cell line and warns that while EA.hy926 cells is helpful in studies of some genes they appear to be less suited for other studies such as those involved in the regulation of cell proliferation and apoptosis.
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1.12 Aims

Both shear stress and thrombin appear to either directly or indirectly exert their effect through similar signal transduction mechanisms in endothelial cells including signalling to focal adhesion proteins and calcium regulated pathways. The aim of the current investigation was to contribute in the understanding of the mechanisms involved to the transduction of these signals by comparing the morphological and signalling responses to shear stress and thrombin.

A major part of the current knowledge on the effects of thrombin and shear stress has come from in vitro experiments carried out in primary cells including human umbilical vein endothelial cells (HUVECs) and bovine aortic endothelial cells (BAECs). To date, there is relatively little information on the effect of thrombin or shear stress on EA.hy926 cells: thrombin has been reported to induce cell rounding in an MLCK and Rac-dependent manner, a response similar to that of HUVECs (Vouret-Craviari et al., 1998), and the expression of carbohydrate sulfotransferases, CHST1 and CHST2 responsible for the sulfation of and thereby the generation of functional L-selectin ligands, has been reported in shear stress treated EA.hy926 cells (Li et al., 2001). In addition, both stimuli have been reported to induce the release of tissue factor pathway inhibitor (TFPI) in EA.hy926 cells, indicating the anticoagulant potential of both thrombin (Lupu et al., 1995) and shear stress (Westmuckett et al., 2000). Part of the aims became therefore, the characterization of EA.hy926 cell responses to thrombin and shear stress and their comparison with primary cells HUVECs and BAECs, in order to determine which responses were common and which varied between the cell types and contribute to the understanding of endothelial cell specificity as well as variability; veins Vs arteries (HUVECs Vs BAECs) and macrovascular Vs macrovascular (HUVECs Vs EA.hy926 cells). Comparisons of the effects of different stimuli on the same cell type aim towards contributing in the understanding of the mechanisms by which two different signals (thrombin and shear stress) utilizing components of the same pathway result in distinct morphological changes.
2 MATERIALS AND METHODS

2.1 Materials

Table 2.1: Materials and Sources: Alphabetical list of materials and their sources. Any unlisted chemicals were obtained from Sigma Aldrich (www.sigmaaldrich.com)

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### Materials and Methods

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<td>Whatman <a href="http://www.whatman.com">www.whatman.com</a></td>
</tr>
<tr>
<td>Glutathione Sepharose</td>
<td>Amersham Biosciences <a href="http://www.amershambiosciences.com">www.amershambiosciences.com</a></td>
</tr>
<tr>
<td>Protein A and protein G-sepharose</td>
<td></td>
</tr>
<tr>
<td>Immobilon-P transfer membrane</td>
<td>Millipore <a href="http://www.millipore.com">www.millipore.com</a></td>
</tr>
<tr>
<td>Media and media supplements</td>
<td>Cambrex <a href="http://www.cambrex.com">http://www.cambrex.com</a></td>
</tr>
<tr>
<td>Microscope Equipment</td>
<td>Chapter 2.2.3</td>
</tr>
<tr>
<td>Mowiol</td>
<td>Calbiochem <a href="http://www.calbiochem.com">www.calbiochem.com</a></td>
</tr>
<tr>
<td>Nitro Blue Tetrazolium (NBT)</td>
<td>Roche Pharmaceuticals <a href="http://www.roche.com">www.roche.com</a></td>
</tr>
<tr>
<td>NP40</td>
<td>Biochemica International <a href="http://www.biochemica.com">www.biochemica.com</a></td>
</tr>
<tr>
<td>Oligofectamine</td>
<td>Invitrogen <a href="http://www.invitrogen.com">www.invitrogen.com</a></td>
</tr>
<tr>
<td>Oligonucleotides</td>
<td>Table 2.9</td>
</tr>
<tr>
<td>Calpain 1 and 2 siRNA</td>
<td>Dharmacon <a href="http://www.dharmacon.com">www.dharmacon.com</a></td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>Invitrogen <a href="http://www.invitrogen.com">www.invitrogen.com</a></td>
</tr>
<tr>
<td>Protein Molecular Weight Markers</td>
<td>Bior-Rad Laboratories Ltd <a href="http://www.biorad.com">www.biorad.com</a></td>
</tr>
<tr>
<td>RPN rainbow molecular weight marker</td>
<td>Amersham Pharmacia <a href="http://www.amershambiosciences.com">www.amershambiosciences.com</a></td>
</tr>
</tbody>
</table>
**Materials and Methods**

<table>
<thead>
<tr>
<th>MATERIAL</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td>American Bioanalytical [<a href="http://www.americanbio.com">www.americanbio.com</a>]</td>
</tr>
<tr>
<td>SDS-PAGE Precast Gel Materials</td>
<td>Invitrogen [<a href="http://www.invitrogen.com">www.invitrogen.com</a>]</td>
</tr>
<tr>
<td>Gels and Mops Running Buffer (X20)</td>
<td>Invitrogen [<a href="http://www.invitrogen.com">www.invitrogen.com</a>]</td>
</tr>
<tr>
<td>Shear Stress Equipment</td>
<td>Table 2.4</td>
</tr>
<tr>
<td>Super RX medical X-ray film</td>
<td>Fuji [<a href="http://www.fujimed.com">www.fujimed.com</a>]</td>
</tr>
<tr>
<td>Sodium Dodecyl Sulphate (SDS)</td>
<td>Invitrogen [<a href="http://www.invitrogen.com">www.invitrogen.com</a>]</td>
</tr>
<tr>
<td>Trypsin/EDTA</td>
<td>Calbiochem [<a href="http://www.emdbiosciences.com">www.emdbiosciences.com</a>]</td>
</tr>
</tbody>
</table>

### Table 2.2: Primary Antibodies

Alphabetically listed by antibody name. Abbreviations: western blotting (WB), Immunofluorescence (IF)

<table>
<thead>
<tr>
<th>Antibody Name (Species)</th>
<th>Catalogue No.</th>
<th>Working Concentrations</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calpain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calpain-1 (rabbit)</td>
<td>C-266</td>
<td>WB: 0.2 µg/ml</td>
<td>Sigma Aldrich [<a href="http://www.sigmaaldrich.com">www.sigmaaldrich.com</a>]</td>
</tr>
<tr>
<td>Calpain-2 (rabbit)</td>
<td>C-268</td>
<td>WB: 0.2 µg/ml</td>
<td></td>
</tr>
<tr>
<td>ERK-1 (rabbit)</td>
<td>Sc-94</td>
<td>WB: 0.05 µg/ml</td>
<td>Santa Cruz Biotech. [<a href="http://www.scbt.com">www.scbt.com</a>]</td>
</tr>
<tr>
<td>FAK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>total (mouse)</td>
<td>61007</td>
<td>WB: 0.25 µg/ml</td>
<td>Transduction Lab. [<a href="http://www.bdbiosciences.com">www.bdbiosciences.com</a>]</td>
</tr>
<tr>
<td>pY(397) (rabbit)</td>
<td>NA</td>
<td>WB: 0.25 µg/ml</td>
<td>Upstate [<a href="http://www.upstate.com">www.upstate.com</a>]</td>
</tr>
<tr>
<td>pY(397) (mouse)</td>
<td>611807</td>
<td>IF: 1.25 µg/ml</td>
<td></td>
</tr>
<tr>
<td>pY(576) (rabbit)</td>
<td>07-157</td>
<td>WB: 0.25 µg/ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>WB: 1 µg/ml</td>
<td></td>
</tr>
</tbody>
</table>
### Materials and Methods

<table>
<thead>
<tr>
<th>Antibody Name (Species)</th>
<th>Catalogue No. (Stock onc.)</th>
<th>Application &amp; Working Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMLC (rabbit) (Ser19/Thr18)</td>
<td>sb12896</td>
<td>WB: 1 µg/ml</td>
<td>SantaCruz Biotechnology <a href="http://www.scbt.com">www.scbt.com</a></td>
</tr>
<tr>
<td>Paxillin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pY(118) (rabbit)</td>
<td>44-722G</td>
<td>WB: 0.05 µg/ml IF: 0.5 µg/ml</td>
<td>Biosource <a href="http://www.biosource.com">www.biosource.com</a></td>
</tr>
<tr>
<td>pY(31) (rabbit)</td>
<td>abs5248</td>
<td>WB: 0.2 µg/ml</td>
<td>Abcam <a href="http://www.abcam.com">www.abcam.com</a></td>
</tr>
<tr>
<td>total (mouse)</td>
<td>610051</td>
<td>WB: 0.05 µg/ml IF: 0.5 µg/ml</td>
<td>Transduction Lab. <a href="http://www.bdbiosciences.com">www.bdbiosciences.com</a></td>
</tr>
<tr>
<td>Racl (mouse)</td>
<td>05-389</td>
<td>WB: 0.05 µg/ml</td>
<td>Upstate Biotechnology <a href="http://www.upstate.com">www.upstate.com</a></td>
</tr>
<tr>
<td>RhoA (mouse)</td>
<td>sc-418</td>
<td>WB: 0.2 µg/ml</td>
<td>Transduction Lab. <a href="http://www.bdbiosciences.com">www.bdbiosciences.com</a></td>
</tr>
<tr>
<td>Src</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (rabbit)</td>
<td>2108</td>
<td>WB: 0.1 µg/ml</td>
<td>Cell Signaling Techn. <a href="http://www.cellsignal.com">www.cellsignal.com</a></td>
</tr>
<tr>
<td>pY(416) (rabbit)</td>
<td>2101</td>
<td>WB: 0.1 µg/ml</td>
<td></td>
</tr>
<tr>
<td>Talin (mouse)</td>
<td>T3287</td>
<td>WB: 0.05 µg/ml IF: 0.4 µg/ml</td>
<td>Sigma Aldrich <a href="http://www.sigmaaldrich.com">www.sigmaaldrich.com</a></td>
</tr>
<tr>
<td>β-Tubulin (mouse) acetylated tubulin (mouse)</td>
<td>T4026</td>
<td>WB: 0.4 µg/ml IF: 1 µg/ml WB: 0.2 µg/ml</td>
<td>Sigma Aldrich <a href="http://www.sigmaaldrich.com">www.sigmaaldrich.com</a></td>
</tr>
<tr>
<td>VE-Cadherin (mouse)</td>
<td>555661</td>
<td>IF: 0.5 µg/ml</td>
<td>Pharmingen <a href="http://www.bdbiosciences.com">www.bdbiosciences.com</a></td>
</tr>
</tbody>
</table>
### Materials and Methods

#### Table 2.3: Secondary Antibodies
Secondary antibodies used for western blotting (WB) or immunofluorescence (IF) experiments.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Application &amp; Working Dilution.</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy5-conjugated-anti - mouse/rabbit</td>
<td>IFL: 2 µg/ml</td>
<td>Jackson Immuno-Research</td>
</tr>
<tr>
<td></td>
<td></td>
<td><a href="http://www.jacksonimmuno.com">www.jacksonimmuno.com</a></td>
</tr>
<tr>
<td>FITC or TRITC - conjugated -anti-mouse/rat</td>
<td>IFL: 2 µg/ml</td>
<td>Southern Biotechnology</td>
</tr>
<tr>
<td></td>
<td></td>
<td><a href="http://www.southernbiotech.com">www.southernbiotech.com</a></td>
</tr>
<tr>
<td>AP - conjugated - anti-rabbit AP: Alkaline Phosphatase</td>
<td>WB 1 µg/ml</td>
<td>Amersham Pharmacia</td>
</tr>
<tr>
<td></td>
<td></td>
<td><a href="http://www.amershambiosciences.com">www.amershambiosciences.com</a></td>
</tr>
<tr>
<td>Horse Radish Peroxidase (HRP) -conjugated-goat-anti -mouse/rabbit</td>
<td>WB 1 µg/ml</td>
<td>Amersham Pharmacia</td>
</tr>
<tr>
<td></td>
<td></td>
<td><a href="http://www.amershambiosciences.com">www.amershambiosciences.com</a></td>
</tr>
<tr>
<td>NON-ANTIBODY REAGENT</td>
<td></td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>TRITC/FITC/Cy5-phalloidin</td>
<td>IFL: 0.2 µg/ml</td>
<td><a href="http://www.sigmaaldrich.com">www.sigmaaldrich.com</a></td>
</tr>
</tbody>
</table>

#### Table 2.4: Shear Stress Equipment
System components applications and sources.

<table>
<thead>
<tr>
<th>System 1</th>
<th>System 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peristaltic Pump</strong> (Masterflex L/S Computerized drive)</td>
<td><strong>Pump</strong> (PHD 22/2000 Syringe)</td>
</tr>
<tr>
<td>Chamber (Polystyrene Slide Flask)</td>
<td>Chamber (μ-slide I: microslide-I) and Silicon Tubing</td>
</tr>
<tr>
<td>Rubber Tubing (PharMed Thermoplastic Masterflex)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Harvard Apparatus</td>
</tr>
<tr>
<td></td>
<td><a href="http://www.harvardapparatus.com">www.harvardapparatus.com</a></td>
</tr>
<tr>
<td></td>
<td>Thistle Scientific</td>
</tr>
<tr>
<td></td>
<td><a href="http://www.thistlescientific.co.uk">www.thistlescientific.co.uk</a></td>
</tr>
<tr>
<td></td>
<td>Masterflex</td>
</tr>
<tr>
<td></td>
<td><a href="http://www.masterflex.com">www.masterflex.com</a></td>
</tr>
<tr>
<td></td>
<td>Nunc</td>
</tr>
<tr>
<td></td>
<td><a href="http://www.nuncbrand.com">www.nuncbrand.com</a></td>
</tr>
<tr>
<td></td>
<td>VWR</td>
</tr>
<tr>
<td></td>
<td><a href="http://www.vwr.com">www.vwr.com</a></td>
</tr>
</tbody>
</table>
**Materials and Methods**

**Table 2.5: Buffers and Solutions**: Alphabetical list of buffers and solutions. Abbreviations: Room Temperature (R.T.), Fresh Prior to Use (FPU).

<table>
<thead>
<tr>
<th>Solution &amp; storage temperature</th>
<th>Recipe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin (100X stock) -20°C</td>
<td>100 mg/ml</td>
</tr>
<tr>
<td>AP buffer +4°C</td>
<td>100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl₂</td>
</tr>
<tr>
<td>APS -20°C</td>
<td>10% (w/v) Ammonium Persulphate</td>
</tr>
</tbody>
</table>

**Blocking Buffers**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCIP (stock) +4°C</td>
<td>50 mg/ml in 100% dimethylformamide</td>
</tr>
<tr>
<td>NBT (stock) +4°C</td>
<td>50 mg/ml in 70% dimethylformamide</td>
</tr>
<tr>
<td>BCIP/NBT FPU</td>
<td>0.33 mg/ml NBT, 0.165 mg/ml BCIP in AP</td>
</tr>
</tbody>
</table>

**Calpain Inhibitor-1 (stock) -20°C** | 25 mg/ml in DMSO |

<table>
<thead>
<tr>
<th>Solution</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calpeptin (stock) -20°C</td>
<td>17 mg/ml in DMSO</td>
</tr>
<tr>
<td>Coomassie Staining Solution R.T.</td>
<td>2.5 mg/ml Coomassie brilliant blue R-250, 40% MetOH (v/v), 10% AcAcid (v/v)</td>
</tr>
<tr>
<td>Coomassie destain solution R.T.</td>
<td>40% Ethanol (v/v), 10% Acetic Acid (v/v)</td>
</tr>
<tr>
<td>DTT (stock) - 20°C</td>
<td>1 M dithiothreitol</td>
</tr>
<tr>
<td>IPTG (stock) -20°C</td>
<td>0.5 M isopropyl β-D-thiogalactopyranoside</td>
</tr>
</tbody>
</table>

**Lysis buffers**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media and media supplements</td>
<td>Table 2.7</td>
</tr>
<tr>
<td>Paraformaldehyde -20°C</td>
<td>4% (w/v) paraformaldehyde in PBS</td>
</tr>
<tr>
<td>PMSF (stock) -20°C</td>
<td>1 M Phenyl methyl sulfonyl fluoride in isopropanol</td>
</tr>
<tr>
<td>Protein Sample Buffer (4X) -20°C</td>
<td>0.25 M Tris pH 6.8, 40% (v/v) glycerol, 10% (w/v) SDS, 2.82 M β-mecraptoethanol, 0.1% (w/v) bromophenol blue</td>
</tr>
<tr>
<td>SDS-PAGE Running buffer (10X stock) R.T.</td>
<td>20 mM glycine, 25 mM Tris-HCl, 0.1% (w/v)</td>
</tr>
<tr>
<td>MOPS (20Xstock) R.T.</td>
<td>50 mM MOPS, 50 mM Tris-Base, 0.1% SDS, 1 mM EDTA pH7.7</td>
</tr>
</tbody>
</table>
## Materials and Methods

<table>
<thead>
<tr>
<th>Solution &amp; storage temperature</th>
<th>Recipe</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SDS-PAGE Gels</strong></td>
<td></td>
</tr>
<tr>
<td>Stalking gel (lower) R.T.</td>
<td>0.5 M Tris HCl (pH 6.8), 0.1% (w/v) SDS, 0.05% (w/v) APS, 0.05% (v/v) TEMED, acrylamide (7%, 12% or 14%)</td>
</tr>
<tr>
<td>Separating gel (upper) R.T.</td>
<td>1 M Tris-HCl (pH 8.8), 4% acrylamide, 1% SDS, 0.5% APS (w/v), 0.1% (v/v) TEMED</td>
</tr>
<tr>
<td>Precast Gel + 4°C, R.T.</td>
<td>3-8%, 4-12%</td>
</tr>
</tbody>
</table>

| SDS-PAGE Transfer buffer      |        |
| (10X stock) R.T.              | 250 mM Tris-HCl, 144 mg/ml Glycine |
| (1X dilution) FPU             | 20% (v/v) MetOH |
| Sodium Azide (1000X stock) + 4°C | 20% (w/v) Sodium Azide |
| Sodium orthovanadate - 20°C   | 100 mM sodium orthovanadate |
| Stripping Buffer R.T.        | 2% (w/v) SDS, 77 mM Tris pH 6.8, 0.68% (v/v) β-mercaptoethanol |
| TBS (20X stock) + 4°C         | 25 mM Tris-HCl pH 7.5, 150 mM NaCl |
| TBS-Tween R.T.               | 20 mM Tris-HCl pH 7.6, 130 mM NaCl, 0.1% Tween-20 |
| Thrombin (stock) - 20°C       | 100 U/ml |
| Transfer Buffer (10X) R.T.   | 20% methanol, 2 mM glycine. 275 mM Tris-HCl |
| TritonX-100 (stock) + 4°C     | 10% (v/v) Triton-X-100 |

### Table 2.6: Blocking Buffers: recipes and applications. Immunofluorescence (IF), Immuno blotting (IB), Primary and secondary antibodies (1° and 2° Abs)

<table>
<thead>
<tr>
<th>Blocking Buffer Recipes</th>
<th>Application: dilution of 1° and 2° Abs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% (w/v) BSA in PBS</td>
<td>IF</td>
</tr>
<tr>
<td>5% (w/v) BSA in TBS-Tween</td>
<td>WB</td>
</tr>
<tr>
<td>5% (w/v) non fat milk in TBS-Tween</td>
<td>WB</td>
</tr>
<tr>
<td>1% (w/v) non fat milk in TBS-Tween</td>
<td>WB</td>
</tr>
</tbody>
</table>
**Materials and Methods**

**Table 2.7: Lysis Buffers:** stock recipes and freshly added inhibitors (FAI)

<table>
<thead>
<tr>
<th>Lysis Buffer</th>
<th>Stock Recipe</th>
<th>FAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP40</td>
<td>50 mM Tris pH 7.6</td>
<td>Protease Inhibitors</td>
</tr>
<tr>
<td></td>
<td>150 mM NaCl</td>
<td>1 mM PMSF</td>
</tr>
<tr>
<td></td>
<td>1 mM EGTA</td>
<td>ApoProtin (1 μg/ml)</td>
</tr>
<tr>
<td></td>
<td>1% (w/v) NP40</td>
<td>Leupeptin (1 μg/ml)</td>
</tr>
<tr>
<td></td>
<td>10 mM MgCl₂</td>
<td>Phosphatase Inhibitors</td>
</tr>
<tr>
<td></td>
<td>Protease Inhibitors</td>
<td>1 mM Na₃VO₄</td>
</tr>
<tr>
<td></td>
<td>1 mM EDTA pH 8.8</td>
<td>1 mM NaF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Disulphide-reducing agents</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5% β-mercaptoethanol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1 mM DTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Where Indicated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Calpain Inhibitors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Calpeptin (17 μg/ml)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Calpain Inhibitor-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(17 μg/ml)</td>
</tr>
<tr>
<td>STE</td>
<td>10 mM Tris-Cl pH 78.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>150 mM NaCl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 mM EDTA</td>
<td></td>
</tr>
<tr>
<td>RheB Rho Extraction Buffer</td>
<td>50 mM Tris-HCl pH 7.6</td>
<td>Protease Inhibitors</td>
</tr>
<tr>
<td></td>
<td>500 mM NaCl</td>
<td>1 mM PMSF</td>
</tr>
<tr>
<td></td>
<td>0.1% (w/v) SDS</td>
<td>ApoProtin (1 μg/ml)</td>
</tr>
<tr>
<td></td>
<td>1% (v/v) TritonX-100</td>
<td>Leupeptin (1 μg/ml)</td>
</tr>
<tr>
<td></td>
<td>10% Glycerol</td>
<td>Phosphatase Inhibitors</td>
</tr>
<tr>
<td></td>
<td>0.5% Na-deoxycholate</td>
<td>1 mM Na₃VO₄, 1 mM NaF</td>
</tr>
<tr>
<td></td>
<td>10 mM MgCl₂</td>
<td>Disulphide-reducing agents</td>
</tr>
<tr>
<td></td>
<td>Protease Inhibitors</td>
<td>0.5% β-mercaptoethanol</td>
</tr>
<tr>
<td></td>
<td>2 mM EDTA pH 8.8</td>
<td>1 mM DTT</td>
</tr>
</tbody>
</table>

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### Materials and Methods

<table>
<thead>
<tr>
<th>Stock Recipe</th>
<th>FAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis Buffer</td>
<td>Protease Inhibitors</td>
</tr>
<tr>
<td>RIP A</td>
<td>1 mM PMSF,</td>
</tr>
<tr>
<td>50 mM Tris-HCl pH 7.2</td>
<td>Leupeptin (1 μg/ml),</td>
</tr>
<tr>
<td>150 mM NaCl</td>
<td>Aprotinin (1 μg/ml)</td>
</tr>
<tr>
<td>1% TritonX-100 or NP40</td>
<td>Pepestatin (1 μg/ml)</td>
</tr>
<tr>
<td>0.1% SDS</td>
<td>Phosphatase Inhibitors</td>
</tr>
<tr>
<td>1% Na-deoxycholate</td>
<td>1 mM Na3VO4, 1 mM NaF</td>
</tr>
<tr>
<td>10 mM MgCl2</td>
<td>Disulfide reducing agents:</td>
</tr>
<tr>
<td>Protease Inhibitors</td>
<td>1 mM DTT</td>
</tr>
<tr>
<td>1 mM EDTA</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.8: Cell Media and Media Supplements**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Media and Media Supplements</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUVEC</td>
<td>Growing Medium: EBM-2 supplemented with growth factors (EBM-1 Bulletkit), Penicillin (100 IU/ml), Streptomycin (100 μg/ml) and 3% FCS. Starving Medium: EBM-2 supplemented with Penicillin (100 IU/ml), Streptomycin (100 μg/ml), 1% FCS</td>
</tr>
<tr>
<td>EA.hv926 cell line</td>
<td>Growing Medium: DMEM supplemented with Penicillin (100 IU/ml), Streptomycin (100 μg/ml) and 10% FCS. Starving Medium: DMEM medium supplemented with penicillin (100 IU/ml) and Streptomycin (100 μg/ml)</td>
</tr>
<tr>
<td>BAEC</td>
<td>Growing Medium: EGM™ MV supplemented with Penicillin (100 IU/ml), Streptomycin (100 μg/ml) and 5% FCS. Starving Medium: EGM™ MV supplemented with Penicillin (100 IU/ml), Streptomycin (100 μg/ml) and 1% FCS</td>
</tr>
</tbody>
</table>
2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Thawing, culturing, freezing down

Cells (10⁶ cells/ml) were removed from liquid nitrogen, defrosted at 37°C, diluted in 10 ml of growing medium and plated on a 75 cm² flask (HUVEC, EA.hy926, BAEC). Fresh medium was applied to the cells every two days. Confluent flasks were passaged by incubating cells with 5 ml of Trypsin/EDTA for 2 min at 37°C. After cell detachment, the cells were centrifuged at 1200 rpm (ALC PK130R centrifuge) for 5 min at 37°C, resuspended in growing medium and plated on the appropriate surface, as dictated by the experiment. Equal numbers of cells were plated per condition per experiment. A haemocytometer (counting chamber) was used for cell counting. Cultures were incubated at 37°C with 5% CO₂.

Primary cells, HUVEC and BAEC, were used up to a maximum of three passages while the EA.hy926 cell line was cultured for a maximum of one month. Most experiments were carried out on cells from the second passage, both for cell lines and primary cells. HUVEC required coating of surfaces prior to plating (10 μg/ml fibronectin, 20 min, 37°C). For cell freezing, a confluent flask was trypsinized. The pelleted cells were resuspended in growing medium (containing 10% DMSO), aliquoted in cryovials and frozen at -20°C. After one hour the cryovials were transferred to −80°C for 24 hours and then to liquid nitrogen for long term storage.

2.2.1.2 Stimulations and Pre-treatments

Before stimulation with thrombin or calcium ionophore, cells were starved for 16 hours. Whenever pre-treatment of cells was required (calpain inhibitors, EGTA), the incubation was carried out at 37°C for 30 min prior to stimulation. The optimal concentration for calpain inhibitors was determined indirectly using calpain activity assay (Chapter 5.3.2), the concentrations for calcium ionophore A23187 and EGTA were used at concentrations of 10 μM and 1.5 mM respectively as cited in the literature (Cao et al., 2001; Schwartz, 1993). Thrombin activity is measured in a number of different ways, including IOWA Unit, WHO Unit, NIH unit or USP unit. Sigma’s thrombin assay procedure is expressed
in NIH units obtained by direct comparison to a NIH Thrombin Reference Standard, where 1 NIH U = 0.324 ± 0.073 μg. Experiments involving stimulation of endothelial cells with thrombin were carried out using 2 NIH U/ml of thrombin activity which corresponds to about 0.648 μg/ml or about 23 μM.

2.2.1.3 Transfections (RNAi)
EA.hy926 cells were plated in full medium, on 6, 12 or 24 well plates, so that 50-70% confluency was reached the next day. Full medium was then replaced with Optimem medium (without antibiotics or serum). Before the final incubation of the cells with the medium, each well was washed twice with Optimem (without antibiotics or serum). Oligofectamine and siRNAs were incubated together in a small volume of Optimem at room temperature for 15 to 20 min before drop-by-drop addition to each well. The amount of medium and oligofectamine used varied depending on the size of the well (according to manufacturer’s instructions). After 24 hours, Optimem medium was replaced with the same amount of growing medium. Experiments were carried out 48 hours after siRNA transfection.

<table>
<thead>
<tr>
<th>siRNAs</th>
<th>Catalogue Number</th>
<th>Working Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>All purchases were made from Dharmacon <a href="http://www.dharmacon.com">www.dharmacon.com</a></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calpain1 (mu-CPN)</td>
<td>D00580401</td>
<td>15 nM</td>
</tr>
<tr>
<td>Calpain2 (m-CPN)</td>
<td>D00579901</td>
<td>15 nM</td>
</tr>
<tr>
<td>csiRNA (control siRNA)</td>
<td>D00121001</td>
<td>20 nM</td>
</tr>
<tr>
<td>siGLO Red-siGLO Green-</td>
<td>D00163002</td>
<td>0.20 nM when co-transfected</td>
</tr>
<tr>
<td>Transfection indicators;</td>
<td>D00163001</td>
<td>20 nM when used alone as control</td>
</tr>
<tr>
<td>stable fluorescent non-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>targeting control siRNA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.9: Concentrations of siRNA and transfection indicators: The table indicates the siRNAs used for siRNA transfection experiments. It includes isoform specific siRNAs, control siRNAs and transfection indicators which are also siRNA bound and were used as controls.
Materials and Methods

For immunostaining and live cell imaging, the transfection complex was formed by incubating oligofectamine with siRNA as well as green or red transfection indicators (Table 2.9). The transfection was carried out in 6 well plates and after 24 hours cells were trypsinized and plated at the appropriate concentrations on cover slips or shear stress chambers. Co-transfection of calpain siRNA with siGLO helped to identify transfected cells by fluorescence but did not necessarily indicate transfection with calpain siRNA or efficient knock-down of the protein. For experiments involving thrombin stimulations, the growing medium was replaced with starving medium 32 hours after transfection (16 hours prior to the beginning of the experiment). Shear stress experiments did not require any starvation. For all experiments involving transfections, the efficiency of the siRNA was determined by western blotting, 48 hours after transfection. A small amount of the cells per condition was reserved for plating on a 24 well plate and lysed in 100 μl of 4X sample buffer. The efficiency of the siRNA was measured against that of siGLO-transfected cells and/or non-treated cells as well as control siRNA-transfected cells.

2.2.1.4 Shear Stress

Shear stress system 1 (Figure 2.1) was used for biochemistry and immunofluorescence. Cells were cultured in 9 cm² slide flasks until confluent then the flask was disassembled. The microslide, on which the endothelial cells were cultured, was used to assemble the shear stress chamber [1]. The chamber was then connected via tubing [2] to the medium reservoir [3] (pre-warmed, 37°C) and to the pump [4]. Depending on the experiment, shear stress of 1.6 dyn/cm² or 12 dyn/cm² was applied on the cells. The required magnitude was achieved by adjusting the flow rate according to the shear stress equation provided by the manufacturer [5]. After treatment, the chamber was disassembled and the microslide placed in a 10 cm dish, on ice. For biochemistry experiments, cells were lysed in 100 μl lysis buffer or sample buffer in accordance with the cell lysis protocol (Chapter 2.2.2.1). Alternatively, treated cells were fixed and immunostained on the microslide (Chapter 2.2.3).
Shear stress system 2 (Figure 2.2) was used for live imaging experiments (Chapter 2.2.3.2). Cells were cultured to monolayer confluency in 6 well plates. On the day before the experiment, cells were trypsinized, pelleted down and re-suspended in 200 µl (per well) of growing medium. In order to obtain a confluent monolayer for the day of the experiment, 100 µl of the cell suspension were transferred onto a microslide-1 chamber and incubated overnight at 37°C. The remaining 100 µl of cell suspension were discarded. Alternatively, they were plated on separate wells in a 24-well plate and lysed the day of the shear stress experiment (Chapter 2.2.1.3). In order to obtain a sub-confluent monolayer, 50 µl of the cell suspension were plated. On the day of the experiment, the microslide [1] was removed from the incubator, connected to the medium reservoir [2] and pump [3], via tubing [4] and placed under the microscope. Depending on the experiment, shear stress of 1.6 dyn/cm² or 12 dyn/cm² was applied on the monolayer [5]. Images were collected and generated as described in (Chapter 2.2.3.2).
2.2.2 Protein preparation and analysis

2.2.2.1 Cell lysis

Different lysis buffers were used depending on the experiment. Lysis buffers were supplemented with inhibitors added fresh before lysis (Table 2.7). Cells were placed with the buffer on ice (500 μl for approximately 1.5 million cells) and immediately transferred to a 1.5 ml cold microfuge tube. The lysates were rotated for 10 min at 4°C before centrifugation (8 min, 4°C, 14 000g). The supernatant was collected and the pellet discarded. 10 μl of the supernatant were removed to calculate the protein concentration of the sample (BioRad assay). The rest of the supernatant was boiled with 4X sample buffer for 10 min at ~ 90°C and stored at -20°C.

Alternatively, after treatment cells were lysed directly in 4X sample buffer. The buffer was heated for 5 min at 100°C prior to use and was applied onto the cells after treatment. Equal volume of sample buffer was used per condition. A 19-G needle and syringe was used to shear the DNA and the sample was then transferred to a 1.5 ml microfuge tube, boiled (10 min at 90°C) and stored at -20°C until required.
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2.2.2.2 Protein concentration
After lysing in NP40 lysis buffer, the protein concentration of each sample was calculated using a standard curve created from known concentrations of BSA. Protein assay reagent (BioRad) was diluted in distilled water (1:20). The standard curve was prepared by adding 2, 4, 8, 16 and 20 mg/ml BSA to the diluted reagent (final volume= 1ml). In parallel, 5 µl of each sample was added to 995 µl of the diluted reagent. All dilutions were prepared in polystyrene cuvettes. After brief vortexing the absorbance at 595 nm was recorder for each sample. Sample protein concentrations were then calculated based on the absorbance at 595 nm of the samples making up the standard curve. For cells lysed in sample buffer it was not possible to determine the protein concentration prior to analysis (Chapter 2.2.5.1).

2.2.2.3 SDS-PAGE and Immunoblotting
Proteins were separated by SDS-PAGE either on a mini apparatus (Mini-Protean II Cell, Biorad) or on pre-cast gels. Depending on the proteins of interest 7%, 12% polyacrylamide gels, or 4-12%, 3-8% gradient gels were used. For RhoA and Rac1 pull-downs a 14% gel was used. The samples were loaded at the same protein concentrations, when the sample concentration could be determined by BioRad assay or equal volumes when cells were lysed directly in sample buffer.

The proteins were transferred from the gel onto an Immobilon-P membrane using semi-wet transfer (90 min at 100 V). After the transfer, the membranes were incubated in blocking buffer (5% BSA or 5% non-fat milk, depending on the antibody) for a minimum of 1 hour. The membranes were then washed three times for 10 min with 0.1% TBS-Tween and incubated with the primary antibody in 1% non-fat milk or 5% BSA and 0.02% sodium azide. The concentration of the antibody (Table 2.2), the blocking and time of incubation varied with the protein under investigation. Membranes were washed three times for 10 min with 0.1% TBS-Tween). The membrane was then incubated with HRP-labeling secondary antibody for 1 hour, washed with 0.1% TBS-Tween, developed with ECL reagents and exposed to X-ray films. When calpain antibodies were used, membranes could not be developed with ECL reagents and required instead incubation
Materials and Methods

with alkaline-conjugated anti-immunoglobulin secondary antibodies for 90 min and were developed using freshly prepared BCIP/NBT solution. The bands were visualized after 1h of membrane incubation with the developing solution followed by thorough rinsing with H$_2$O.

Some membranes were stripped to allow re-probing with different antibodies. The membranes were incubated with stripping buffer for 20 min at 65°C, with TBS/0.5% Tween for a further 20 min and a final wash with 0.1% TBS-Tween for 10 min. The membranes were then re-blocked and re-probed with different primary antibodies. Re-probing was not possible for membranes developed using BCIP and NBT.

2.2.2.4 Analysis of RhoA and Rac1 activity

*E. Coli* BL21 strains transformed with pGEX-2T encoding GST-RhoetekinRBD (lab construct from Dr. John Collard, Netherlands Cancer Institute) or GST-PBD (lab construct from Dr. Martin Schwartz, Scrippes Institute) for assaying Rho and Rac activities respectively, were grown overnight in 100 ml LB medium supplemented with 100 µg/ml ampicillin. The culture was diluted in fresh ampicillin-containing LB medium (1:20) and grown until O.D ~ 0.8 was reached ($\lambda=600$ nm). At this point 50 ml of the culture was removed and placed on ice in order to serve as a control for IPTG induction. IPTG (0.45 mM) was added to the rest of the culture and cells were incubated for a further 2 hours at 30°C. The mixture was aliquoted into 50 ml Falcon tubes and the bacteria were pelleted by centrifugation (20 min, 4°C, 3000 rpm using Beckman centrifuge J-6M/E). The bacterial pellets of the control were also obtained. The pellets were kept at -80°C prior to purification of GST-RBD or GST-PBD

When required, the pellets were placed on ice and re-suspended in cold STE buffer (0.75 ml buffer per pellet) supplemented with 1 mM PMSF. The mixtures were homogenized with a 19-G needle and syringe, and lysed in 100 µg/ml of lysozyme (freshly dissolved in STE buffer). The mixture was incubated with lysozyme on ice for 15 min with occasional mixing. Subsequently, 5 mM DTT, 1% Tween-20 and 0.03% SDS were added to the mixture. After each addition, the lysate was mixed by (hand-) rocking 8 to 10 times. The
resulting viscose lysates were aliquoted in 1.5 ml microfuge tubes and centrifuged for 30 min at 14 000 g at 4°C. 40 μl of the lysate were removed and electrophorressed on a 14% polyacrylamide gel along with the lysate obtained from the IPTG control. The gel was stained with Coomassie blue to check the induction of protein expression. If the induction worked, the lysates were transferred to a fresh tube and incubated with a 50% pre-washed (STE buffer, 3 washes) slurry of glutathione sepharose beads, for 15 min at room temperature. The beads were then collected and washed with ice cold STE buffer three times. After washing, the beads were resuspended in STE buffer, 50% (v/v) and stored at 4°C to be used the next day. 10 μl of the bead suspension was removed, boiled with 5 μl sample buffer and electrophorressed on a 14% polyacrylamide gel along with known concentrations of BSA, in order to estimate amounts of purified GST-fusion proteins by Coomassie blue staining (Figure 2.3).

<table>
<thead>
<tr>
<th>Beads</th>
<th>mg/ml BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBD RBD</td>
<td>2 4 8 16 32</td>
</tr>
</tbody>
</table>

**Figure 2.3: Quantification of GST-fusion proteins.** Beads containing GST-PBD or GST-RBD of unknown concentrations were electrophorressed on a 14% polyacrylamide gel against the indicated concentrations of BSA. Based on the bands intensity revealed from Coomassie blue staining (Chapter 2.2.2.5), the sample concentrations were estimated.

For pull-down assays, cells were cultured on 10 cm dishes. After treatment, the cells were lysed on ice using 500 μl RheB with inhibitors added fresh prior to use (Table 2.7). The lysates were immediately centrifuged for 5 min at 14 000 g at 4°C. A small aliquot (40 μl) of the supernatant was then removed and boiled in sample buffer. The rest of the supernatant was incubated with 40 to 45 μg of GST-PBD or GST-RBD on beads per reaction, on a rotating wheel at 4°C for 90 min. The beads were then centrifuged (30 sec, 4°C, 14 000 g), washed three times with RheB buffer (without inhibitors) and boiled in 20 μl of 2X sample buffer. The pull-down samples and the samples containing the total Rho protein were electrophorressed on a 14% polyacrylamide gel, transferred to Immobilon-P membranes and probed with Rho protein antibodies (Rac1 or RhoA.
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depending on the experiment). The samples containing total Rho protein levels served as a loading control.

2.2.2.5 Coomassie Blue staining of SDS-polyacrylamide gels
The gel was removed from the gel apparatus and transferred to a sealed container containing 100 ml staining solution. After 20 to 30 min of incubation (room temperature, shaking) the staining solution was replaced by destain solution. The gel was incubated for 10 min (room temperature, shaking) with destain solution. After two additional 10 min washes with fresh destain solution, bands were visualized and scanned using a BioRad GS-800 densitometer.

2.2.3 Immunostaining and image generation
2.2.3.1 Immunofluorescence and confocal microscopy
After treatment, cells were fixed on coverslips or shear stress micro-slides, permealised via incubation with 0.2% Triton X-100 for 5 min and blocked for 1 hour at room temperature (or for 16 hours at 4°C) with 1% BSA. The method of fixation varied depending on the primary antibody used. Talin antibody required methanol fixation. Coverslips were incubated at -20°C with 100% (ice-cold) methanol for 15 min. For all other antibodies, cells were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature. Primary antibodies were diluted in 1% BSA/PBS at an optimal concentration as determined by titration or according to manufacturers’ instructions (Table 2.2). Cover slips or shear stress micro-slides were incubated with the primary antibody for 1 hour at room temperature (or 16 hours at 4°C), followed by incubation for 1 hour with the secondary antibody at room temperature. Triple staining was usually carried out, by using different secondary antibodies for each primary antibody (TRITC-conjugated, FITC-conjugated, Cy5-conjugated antibodies or phalloidin). Before and after each step, cells were washed several times with PBS. Coverslips and shear stress micro-slides were mounted using Mowiol anti-quench agent containing 0.1% phenylene-diamine.
Materials and Methods

Cells were visualized by Zeiss LSM laser-scanning microscope (www.zeiss.com) using a 40X/1.30 NA oil immersion objective and LSM 510 image recording software. Image files were collected as a matrix of 1024 X 1024 in an LSM format and exported as TIFF images (16-bit) for further image manipulation using Photoshop.

2.2.3.2 Time-Lapse Microcopy

EA.hy926 cells cultured in 10 cm dishes were allowed to reach confluency before starving for 16 hours prior to their visualization under a time-lapse microscope. A heated stage and air heating were used to maintain the temperature at 37°C. The chamber was humidified using a water-wall and carbon dioxide was maintained at 5%. Cells were visualized in the absence and presence of inhibitors using phase-contrast microscopy for 30 min after which visualization continued for one further hour after the addition of the stimulus (thrombin). Frames were collected with a KPM1E/K-S10CCD camera (Hitachi Denshi, Japan www.hdai.com) using a Zeiss Axiovert 135 microscope (www.zeiss.com) and X20 air objective. Frames were collected every 60 sec over a period of 90 min.

For shear stress time-lapse experiments that involved transfection of cells with transfection indicators, frames were collected 60 min using a Nikon Eclipse TE250-E Microscope (www.nikon-instruments.com) with combined TRITC/Phase configuration and X20 air objective. Frames were collected every 30 sec alternating between the two phases. Cells were cultured in HEPES/Hanks’ balanced salts based medium to allow correct pH buffering in air during the collection of the time series.

For all time-lapse experiments images were collected using Andor IQ software technology, saved as individual TIFF files and movies were re-created using Image J software (http://rsb.info.nih.gov/ij).

2.2.4 Calpain activity assays

Calpain activity assays were carried out using a calpain activity assay kit. The assay is a fluorogenic one which measures the activity of calpain 1 and 2 based on the detection of cleavage of a synthetic calpain substrate (Ac-LLY-AFC). AFC is released upon cleavage.
by calpain and is measured fluorometrically at an excitation wavelength (485 nm) and an emission wavelength (530 nm).

Cells were plated at 1-2 X10^6 in growing medium, on 6 cm dishes. After 24 hours, cells were starved (16 hours prior to stimulation). After treatment, cells were lysed in 100 μl of extraction buffer and incubated on ice for 20 min with occasional mixing. The lysates were then centrifuged (1 min, 10,000g) and the supernatant was transferred to a fresh tube on ice. 10 μl of the lysate was used in order to assay protein concentration for each condition (BioRad assay). A sample volume containing approximately 100 μg of protein was diluted to 85 μl with extraction buffer and transferred in 96 well black microplates. For a positive control, 1 to 5 μl of active calpain diluted to 85 μl with extraction buffer was used. Highly purified calpain 1 from human erythrocytes was provided in the kit. Untreated cell lysates, and lysates of cultures treated with 1 mM EGTA (calcium chelator) were used as negative controls. As an additional negative control, 1 μl Calpain Inhibitor (Z-LLY-FMK) was added to the cell lysate. 10 μl of 10X Reaction Buffer and 5 μl of calpain substrate was added to each condition. The samples were then incubated at 37°C for 1 hour in the dark. Then, quantification of the fluorescence of each condition was carried out using a fluorescence plate reader (α fusion, Perkin-Elmer timer). The changes in calpain activity were determined relative to that of untreated cells after subtracting the fluorescence values obtained from lysates in which the calpain specific substrate was not added (background).

2.2.5 Quantifications
2.2.5.1 Western Blotting
Developed X-ray films were scanned using a BioRad GS-800 densitometer and the density of each band was determined, both for the protein of interest as well as the loading control. The values were then exported to Microsoft Excel for further analysis: Samples were corrected for loading by comparing with their respective loading controls. Fold changes of the protein of interest were determined with respect to control experiments. Statistical analysis was performed in order to determine the statistical significance of the observed changes (Chapter 2.2.5.3).
Materials and Methods

2.2.5.2 Cell Area and Circularity
As discussed previously, thrombin has been shown to induce endothelial cell contraction whereby cells become more rounded and their spread area decreases. Cell size and circularity measurements were therefore used as means of investigating the effect of specific treatment to the thrombin-induced contraction of endothelial cells. Image J software was used to quantify all parameters from TIFF images generated by confocal microscopy or time lapse movies. An outline was automatically drawn around the perimeter of single cells and a threshold number was set to eliminate background fluorescence. Setting the threshold was a manual process that involved trial and error in order ensuring that only background was eliminated. Cell area (in pixels) and circularity were calculated automatically by the software. Circularity was given as a number between 0 and 1 with 1 denoting a perfect circle. When measuring confluent images, single cells were outlined by manually removing areas of cell-cell attachment using the brush tools. Area and circularity parameters were obtained for a minimum of 30 cells per condition in each of at least three independent experiments. Statistical analysis was performed comparing treated cells with controls in order to determine the statistical significance of the observations (Chapter 2.2.5.3).

2.2.5.3 Statistical Analysis
Statistical analysis was carried out wherever data were available from at least three independent experiments in order to determine whether the changes observed in response to a treatment were significant. Statistical significance was calculated using Student’s t-test and ANOVA (analysis of variance). Statistical significance and standard error calculations were automated using Microsoft Excel software. Two values were considered statistically different if statistical significance was indicated by both tests.
3 The effect of thrombin and shear stress on the cytoskeleton

3.1 Introduction

Maintaining the integrity of the endothelial monolayer is important as the vascular endothelium plays a significant role in many physiological processes, while many pathological processes have been reported to implicate its disruption (Ryan, 1988). The integrity of the endothelium depends on cell-cell adhesions (Seebach, 2005) and the cytoskeleton (Bogatcheva, 2001).

It is now well established that endothelial cells respond to both thrombin and shear stress by altering their morphology; a process that requires remodeling of both the actin and the microtubule cytoskeleton (Bogatcheva, 2001; Smurova, 2004; Wotjciak-Stothard, 2003). In addition to the cytoskeleton, thrombin and shear stress have been reported to influence cell-cell adhesion molecules such as VE-cadherin (Miao 2005; Tzima et al., 2005; Flemming et al., 2005). Both of these responses involve Rho GTPases.

The effects of shear stress and thrombin on the actin and microtubule networks as well as VE-cadherin and activation of RhoA and Rac1 were investigated in EA.hy926 cells and in HUVECs. The immortalized cell line, EA.hy926, is a fusion of HUVEC with A459 lung carcinoma epithelial cells. It constitutes one of the best characterized endothelial cell lines and possesses most of the phenotypic endothelial cell characteristics (Bouis et al., 2001). Experiments were carried on both HUVECs and EA.hy926 cells allowing for comparisons of endothelial responses between different stimuli and different cell types.
3.2 The effect of thrombin and shear stress on actin cytoskeleton

The effect of thrombin and shear stress on the actin cytoskeleton was investigated as both stimuli had previously been reported to induce actin reorganization in endothelial cells (Bogatcheva, 2001; Wojciak-Stothard, 2003).

3.2.1 The effect of thrombin on actin reorganization

Thrombin was induced some cell contraction (cell rounding) in HUVECs as well as in EA.hy926 cells (Figure 3.1). The effect was strong with initial cell contraction observed as early as 10 min after stimulation and progressively increasing to 60 min. Cell contraction involved reorganization of the actin cytoskeleton associated with increased amounts of cortical stress fibres in both EA.hy926 cells and HUVECs. Endothelial cell contraction and stress fibre reorganization also occurred in EA.hy926 cells exposed to thrombin concentrations down to 0.5 U/ml for the time period of 10 min.

At the 60 min time-point, EA.hy926 cells were observed to increase their stress fibre content spanning the cell and reduce the level of cortical stress fibres. Whether this was the result of reorganization of existing filaments or whether it involved the formation of new stress fibres was not determined.

3.2.2 The effect of shear stress on actin reorganization

In contrast to the thrombin response, endothelial cell contraction did not occur in response to shear stress. In HUVEC, an increase in the numbers of stress fibres spanning the cell was observed to be induced after 30 min of low shear stress application and maintained at 60 min (Figure 3.2b). Interestingly, the adaptive response to flow was different not only in EA.hy926 cells but also in HUVECs, exposed to low (Figure 3.2a) and high (Figure 3.2c) shear stress levels respectively. A reduction in stress fibre content was observed after 60 min both in EA.hy926 cells and HUVECs exposed to low and high shear stress respectively.
Figure 3.1: Endothelial cell response to thrombin stimulation

Confuent endothelial cells were stimulated with the indicated concentrations of thrombin for the specified time-periods. After treatment, cells were fixed and stained for F-actin. 

a) EA.hy926 cells stimulated with 2 U/ml thrombin for the indicated time-periods. Representative of at least three experiments. 

b) HUVECs stimulated with 2 U/ml thrombin or the indicated time-periods. Representative of at least three experiments. 

c) EA.hy926 cells stimulated with the indicated thrombin concentrations for 10 min. Representative of two experiments. Bar=20 μm.
Figure 3.2: Endothelial cell response to shear stress application

Confluent endothelial cells were exposed to the specified magnitudes of shear stress. After treatment, cells were fixed and stained for F-actin. a) EA.hy926 cells exposed to 1.6 dyn/cm² shear stress for the indicated time-periods. b) HUVECs exposed to 1.6 dyn/cm² shear stress for the indicated time-periods. c) HUVECs exposed to 12 dyn/cm² shear stress for the indicated time-periods. All results are representative of three experiments. Bar=20 μm. The black arrow indicates flow direction. Uneven shading across images is the consequence of shear stress chambers not being entirely flat hence affecting image generation.
The effect of thrombin and shear stress on the cytoskeleton

Some degree of cell flattening was observed in HUVEC after 60 min of high shear stress application. Nevertheless, cell flattening could have resulted due to the presence of fewer cells as a consequence of force-induced cell detachment. A certain amount of cell detachment occurred every time stress was applied to a monolayer of EA.hy926 or HUVECs, with more cells detaching under higher flow. A significant amount of cell detachment occurred in EA.hy926 cells exposed to high flow as early as 15 min after shear stress application increasing with time. Hence, the effect of high shear stress on EA.hy926 cells was not analyzed.

Thus, actin reorganization was induced in response to both thrombin and shear stress. While a similar response was exhibited by the two cell types in response to thrombin, cell-type specificity was observed in endothelial cell adaptation to shear stress. Endothelial response to flow was also dependent upon shear stress force.

3.3 The effect of thrombin and shear stress on MLC phosphorylation

The effect of thrombin and shear stress on the phosphorylation of MLC protein was investigated as MLC has been reported to be involved in the regulation of cell contractility and the formation of stress fibres (see Chapter 1.8.2). Increased MLC activity was detected using a phospho-specific antibody recognizing phosphorylated Ser19 and Thr18. MLC kinase is the enzyme reported to be responsible for the phosphorylation of these two sites (Burridge and Chrazowska-Wodnika, 1996).

3.3.1 The effect of thrombin on pMLC

Levels of phosphorylated MLC protein were observed to increase only slightly (10 to 20%) in response to thrombin (Figure 3.3a). Induction of phosphorylation occurred at 2 min after thrombin stimulation and was maintained at levels higher than the control throughout the time course. Even though thrombin-induced increases in pMLC levels were consistent between three independent experiments the small percentage differences meant that changes were not statistically significant.
The effect of thrombin and shear stress on the cytoskeleton

Figure 3.3: Effect of thrombin and shear stress on MLC phosphorylation
Confluent endothelial cells were exposed to thrombin or shear stress for the time-periods indicated. Cells were lysed in sample buffer, electrophorressed on a 4-12% SDS polyacrylamide gel and probed with antibody to pThr18Ser19-MLC. ERK served as loading control. a) EA.hy926 cells stimulated with 2 U/ml of thrombin b) EA.hy926 cells exposed to 1.6 dyn/cm² shear stress. c) HUVECs exposed to 1.6 dyn/cm² shear stress. d) HUVECs exposed to 12 dyn/cm² shear stress. e) pMLC levels of treated cells relative to pMLC levels of control cells. Mean of three independent experiments for each condition +/- s.e.m. with the exception of shear stress treated EA.hy926 cells where only two experiments were carried out. The upper band of ERK was used as a loading control for quantifications. Statistical significance was determined using ANOVA and Student’s t-test (* p<0.05).
3.3.2 The effect of shear stress on pMLC
In contrast to thrombin, pMLC levels in EA.hy926 cells were maintained at control levels throughout the time-course in response to 1.6 dyn/cm² shear stress (Figure 3.3b). Yet, low shear stress was observed to increase pMLC levels in HUVECs significantly (Figure 3.3c). The small (10 to 20%) increase induced after 5 min was observed to reach statistically significant levels (~50%) by 60 min of HUVECs exposure to low shear stress. Interestingly, in HUVEC exposed to high shear stress, levels of pMLC remained constant, similar to the effect of low shear stress application on EA.hy926 monolayers.

Thus, while shear stress-induced formation of stress fibres (HUVEC, 1.6 dyn/cm²) appeared to be associated with significant increases in MLC phosphorylation, thrombin-induced cell contraction was characterized by only minor (if any) changes in pMLC levels within the time-course investigated. The lack of actin reorganization in response to shear stress stimulation (EA.hy926 cells, 1.6 dyn/cm² and HUVEC, 12 dyn/cm²) (Figure 3.2) correlated with the lack of change in pMLC levels throughout the time-course (Figure 3.3).

3.4 The effect of thrombin on RhoA and Rac1 activity
Regulation of actin reorganization MLC phosphorylation status has been reported to occur through the action of small Rho GTPases (see Chapter 1.8.2). MLC phosphorylation has been mostly connected to the function of RhoA, it is also possible that other members of the Rho GTPase family are involved in its regulation (see Chapter 1.8.2); Rac1 activation has been associated with increasing stress fibres in HUVECs (Wojciak-Stothard, 1998). While thrombin is known to activate RhoA in HUVECs (van Nieuw Amerongen et al., 2000; Wojciak-Stothard et al., 2001) its effect in EA.hy926 cells has not yet been reported. The effect of thrombin on RhoA and Rac1 activity was therefore investigated in EA.hy926 cells.
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Figure 3.4: Thrombin induces activation of RhoA but not Rac1
Confluent monolayers of EA.hy926 cells treated with thrombin (2 U/ml) for the time periods indicated. Lysates were incubated with glutathione sepharose beads coupled to a) GST-Rhotekin-RBD or b) GST-PBD to precipitate RhoA-GTP and Rac1-GTP respectively. Precipitates (RhoA-GTP, Rac1-GTP) were electrophorressed on a 14 % SDS polyacrylamide gel in parallel to whole cell lysates (total RhoA and Rac1) and probed with antibodies to a) RhoA and b) Rac1. Equal volumes of whole cell lysates were taken for each condition and are shown in the bottom panels of a and b (total). Results are representative of four experiments. c) Fold intensity values for each time point relative to time-point zero. Average of three experiments. Statistical significance was calculated using ANOVA (p<0.05).
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The activity of RhoA (Figure 3. 4a) was observed to increase after 5 min of thrombin stimulation and maintained up to 30 min. In contrast, thrombin stimulation did not induce activation of Rac1 (Figure 3.4b). Active RhoA levels returned to basal levels after 60 min of thrombin stimulation. Interestingly, this decrease was associated with decreased levels in total RhoA. The decrease in RhoA-GTP activity could therefore be the result of lower expression of the protein rather than induced down-regulation of its activity.

Thus, although these observations do not necessarily exclude the possibility that Rac1 is involved in regulating MLC phosphorylation or actin reorganization, only RhoA was observed to be activated in response to thrombin. For shear stress experiments, it is necessary to culture cells in large chambers due to the large number of cells required for the assay. Unfortunately, for unknown reasons, EA.hy926 cells were not able to survive the culturing process and hence the activity of Rho GTPases was not analyzed in EA.hy926 cells exposed to shear stress.

3.5 The effect of thrombin and shear stress on microtubule cytoskeleton

The effect of thrombin and shear stress on the microtubule reorganization and stability was investigated as both stimuli had previously been reported to induce remodeling of the microtubule cytoskeleton (Bogatcheva, 2001; Wojciak-Stothard, 2003). The effect of thrombin on microtubule stability was investigated by observing the effect of stimulation on the level of acetylated tubulin. Increased microtubule stability is indicated by increased acetylation of the tubulin monomers (see Chapter 1.8.3).

3.5.1 Thrombin-induced reorganization of the microtubule network

Microtubule reorganization occurred in response to thrombin stimulation, associated with a loss of peripheral microtubules (Figure 3.5a). Thrombin-induced reorganization of the microtubule network was associated with a reduction in the level of acetylated tubulin (Figure 3.5b and c) indicating that thrombin destabilizes the microtubules by regulating either the acetylation or the deacetylation processes. The reduction was proportional to time of thrombin incubation and decreased levels ranged from 10 to 40%.
Figure 3.5: The effect of thrombin and shear stress on the microtubule network

a) Confluent EA.hy926 cells were stimulated with thrombin (2 U/ml) for the time-periods indicated, fixed and stained for β-tubulin. b) Confluent EA.hy926 cells were stimulated with thrombin (left panel) or exposed to 1.6 dyn/cm² shear stress (right panel). After treatment cells were lysed in sample buffer, electrophorressed on a 4-12 % SDS-PA gel and probed for acetylated tubulin. Results are representative of three and two experiments for thrombin and shear stress respectively. Erk was used as a loading control. c) Levels of acetylated tubulin of treated cells over acetylated tubulin levels of control +/- s.e.m. Statistical significance for thrombin stimulation was determined using ANOVA and Student’s t-test (* p<0.05)
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3.5.2 Shear Stress induced reorganization of the microtubule network

In contrast to thrombin, shear stress had no effect on microtubule acetylation in EA.hy926 cells (Figure 3.5b and c). Since only two experiments were used for the quantification, significance levels could not be determined.

On the other hand, significant reductions in acetylated tubulin were observed in HUVECs after exposure to 1.6 dyn/cm² shear stress (Figure 3.6a). Shear stress in HUVECs induced a 40% reduction in levels of acetylated tubulin after 10 min which was maintained throughout the time-course. Yet, shear stress did not induce any clear change to the microtubule network or the microtubule organizing center (MTOC).

Similarly, high shear stress did not affect the microtubule network of HUVECs and had no obvious effect on the localization of MTOC (Figure 3.6b). In response to 12 dyn/cm² shear stress levels of acetylated tubulin were maintained around control levels.

Thus, these results suggest that both thrombin and shear stress induce destabilization or increase the turnover of the microtubule filaments, without necessarily affecting the overall organization of the microtubule network. The ability of shear stress to induce tubulin deacetylation was dependent on cell type and shear stress magnitude.
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Figure 3.6: The effect of shear stress on microtubules in HUVECs
Confluent monolayers of HUVECs were exposed to a) 1.6 dyn/cm\(^2\) shear stress or b) 12 dyn/cm\(^2\) shear stress. The black arrows indicate the direction of flow. After treatment cells were fixed and stained for β-tubulin (top panels), or lysed in sample buffer, electrophorressed on a 4-12 % SDS-PAGE gel and probed with antibodies for acetylated tubulin (bottom left panel). ERK was used as a loading control. All results are representative of three experiments. The bottom right panel shows levels of acetylated tubulin in treated cells relative to acetylated tubulin levels of control cells +/- s.e.m. Statistical significance was determined using ANOVA and Student’s t-test (* p<0.05). Uneven shading across images is the consequence of shear stress chambers not being entirely flat hence affecting image generation.
3.6 Junctional Organization

According to published results both thrombin and shear stress affect junctional organization by influencing cell-cell adhesion molecules such as VE-cadherin (Miao, 2005). The effect of thrombin on VE-cadherin localization was therefore investigated in thrombin-stimulated EA.hy926 cells and HUVECs. The same experiment was carried out in cells exposed to shear stress, but due to poor quality of antibody staining on the plastic shear stress chambers no firm conclusions could be made.

3.6.1 The effect of thrombin on junction organization

In unstimulated cells VE-cadherin is predominantly at cell-cell junctions. Even though irregular VE-cadherin staining was present in areas of cell-cell attachment in both stimulated HUVECs and EA.hy926 cells, due to the thrombin-induced cell contraction cells pulling apart were observed to lose junctional staining without a clear increase in intracellular VE-cadherin. Interestingly, after 30 min of thrombin stimulation VE-cadherin appeared more linear at cell-cell junctions than unstimulated controls. This effect was consistent between independent experiments with HUVECs but differed between experiments for EA.hy926 cells. Co-staining of HUVECs with VE-cadherin and F-actin (Figure 3.7b) indicated localization of cortical stress fibres near VE-cadherin under control conditions. The loss of VE-cadherin staining in areas of the cell periphery in response to thrombin correlated with an increase in the level of stress fibres localized at the periphery.
Figure 3.7: The effect of thrombin on VE-cadherin localization

a) Confluent EA.hy926 cells were stimulated with 2 U/ml thrombin for the time-periods indicated and stained for VE-cadherin. Representative of three experiments.

b) Confluent HUVECs were stimulated with 2 U/ml thrombin for the time-periods indicated and stained for VE-cadherin and F-actin. Representative of three experiments.

Bar= 20 μm
3.7 Discussion

3.7.1 The response of endothelial cells to thrombin

The effect of thrombin on the endothelial cytoskeleton and the involvement of small Rho GTPases has been studied in several types of endothelial cells including HUVECs. The effect of thrombin on EA.hy926 cells has not yet been described. For this reason, experiments on HUVECs investigating the effect of thrombin were restricted to morphological changes associated with the actin network and the junctional protein VE-cadherin, to allow comparison with EA.hy926. Nevertheless a more extensive investigation of the effects of thrombin on HUVECs would have been ideal, to enable further comparison between the two cell types. The thrombin concentration used here was 2 U/ml, yet thrombin induced cell contraction even when EA.hy926 cells were incubated with lower concentrations (0.5 and 1 U/ml). Future investigations of the effect of lower thrombin concentrations on EA.hy926 cells would allow a more accurate comparison between these experiments and published results where lower concentrations were used.

Goeckeler and Wysolmerski (1995) reported a rapid sustained isometric contraction of HUVEC monolayers in response to thrombin. The contraction was quantified and found to be 2- to 2.5- fold compared to untreated controls, within 5 min of stimulation and remained elevated for at least 60 min. These results agree with observations of the current investigation for HUVECs, which underwent rapid contraction within 5 of stimulation with thrombin, an effect that was sustained until the last time point, 60 min. Contraction also occurred after 5 min of thrombin stimulation in EA.hy926 cells. Even though the effect was not as strong when compared to HUVECs, it was sustained throughout the time course.

Thrombin-induced contraction leading to gap formation has long been associated with a rearrangement of the actin cytoskeleton and an increase in stress fibre content at the cell periphery as observed here with HUVECs and EA.hy926 cells. Van Nieuw Amerongen et al. (2000) reported these changes in HUVEC responding to 1 U/ml of thrombin and
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investigated the role of small Rho GTPases, RhoA and Rac1. They reported that thrombin induced the activation of RhoA but had no effect on Rac1 activity. This agrees with the data obtained from experiments in EA.hy926 cells reported here where thrombin induced activation of RhoA but not of Rac1, indicating that similar mechanisms are probably involved in regulating the thrombin-induced morphological changes in the two cell types. In HUVECs, the increase in RhoA activity was transient with initial induction observed within 30 seconds of thrombin stimulation followed by a reduction in levels of active RhoA, which nevertheless remained higher than control after 30 min of stimulation (van Nieuw Amerongen et al., 2000). In EA.hy926 cells, increased levels of RhoA-GTP were observed at 5 min, the earliest time-point used for investigating thrombin-induced RhoA activity. It would be interesting to test thrombin-induced RhoA activation at earlier time points in order to compare better the HUVEC and EA.hy926 cell responses. Even though by 60 min of thrombin stimulation RhoA activity had reached basal levels in EA.hy926 cells, this decrease was associated with a reduction in total RhoA levels. Supplementation of these results with the addition of a loading control to test whether the equal volumes of whole cell lysates were also equal in protein content would indicate whether changes in RhoA expression in response to thrombin would be worth investigating.

Even though thrombin-induced Rac1 activity has not been observed in HUVECs (Van Nieuw Amerongen et al., 2000) or EA.hy926 cells, Rac1 has been reported to play a role in regulating endothelial barrier function (Wojciak-Stothard, 2001; Hordijk, 2006). The cell surface glycoprotein vascular adhesion molecule-1 (VCAM-1), whose expression is increased in thrombin-stimulated HUVEC (Minami et al., 2002) has been reported to induce Rac1 activity by a mechanism involving reactive oxygen species (Hordijk, 2006). This VCAM-1-mediated increase in Rac1 activity is associated with increased permeability thereby having an important role in facilitating paracellular transendothelial migration of leukocytes. Nevertheless, increased VCAM-1 expression is a long term effect of thrombin.
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Dominant negative Rac1 increased permeability in untreated HUVEC and this effect was enhanced in the presence of thrombin (Wojciak-Stothard et al. 2001). Inhibition of contractility was also reported as an effect of RhoA inhibition. Although not a conclusion of the group, whose main focus was the role Rho GTPases in permeability, their data complements well with the observations of enhanced RhoA and unaffected Rac1 activities observed in thrombin-stimulated EA.hy916 cells.

Pervanadate (PV), a stimulant which inhibits tyrosine phosphatases thereby inducing the phosphorylation of tyrosine residues, results in endothelial gap formation. PV-stimulated HUVECs, has been reported to induce reorganization of VE-cadherin associated with an early induction in Rac1 activity (Seebach et al., 2005). The partial localization of filamentous actin with an irregular pattern of VE-cadherin staining in untreated HUVEC reported by Seebach et al. (2005) agreed with observations of in the current investigation. Although co-staining of VE-cadherin with F-actin was not carried out in EA.hy926 cells, prevalence of irregular VE-cadherin was observed at cell-cell junctions. Observations between the two investigations also agree with respect to the continuous increase in formation of actin filaments proportional to time of treatment of HUVEC with the respective stimulus and the absence of further co-localization of F-actin with VE-cadherin.

The recruitment of cortical F-actin to cell junctions accompanied by reorganization of VE-cadherin from an irregular to a continuous localization along cell borders was reported after 10 min of HUVECs treatment with PV (Seebach et al., 2005). The transition of VE-cadherin staining from irregular to linear/continuous pattern was observed in thrombin-stimulated HUVEC but only after 30 min of stimulation. To a much lesser extent and not as consistently, the same effect was observed in EA.hy926 cells after 30 min incubation with thrombin. The reorganization of VE-cadherin was reported to relate to PV-induced transient activation of Rac1 (Seebach et al., 2005). As mentioned before, Rac1 activation did not occur in HUVECs in response to 1 U/ml thrombin (Van Nieuw Amerongen et al., 2000), yet it would be interesting to investigate
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The effect of 2 U/ml of thrombin on Rac1 activity in HUVECs and determine whether their thrombin response differs in that respect to that of EA.hy926 cells.

Stress fibre formation and increased actomyosin contractility mediated by phosphorylation of MLC provide the main contribution to the contractile cell response (Bogatcheva et al., 2001). Phosphorylation of MLC is important for contraction in smooth muscle cells but has also been reported to be involved in contraction occurring at cell margins in endothelial cells (Bogatcheva et al., 2001). Exactly which Rho GTPase is responsible for the phosphorylation of myosin light chain (MLC) in endothelial cells is currently under debate, but the strongest evidence indicate that RhoA regulates MLC phosphorylation.

Inhibition of the MLCK, the enzyme responsible for MLC phosphorylation, blocked thrombin induced cell rounding in HUVEC, hence demonstrating that MLC phosphorylation plays an active role in thrombin-induced endothelial cell contraction (Vouret-Craviary et al., 1998). Further more, stimulation of HUVECs with thrombin or histamine increases endothelial permeability, which is associated with increasing levels of MLC phosphorylation (van Nieuw Amerongen et al., 1998). The group reported an early induction of MLC phosphorylation, within 1 min of stimulation and a decrease in the levels after 10 min. They demonstrated that treatment of HUVECs with C3 transferase, which inhibits RhoA activation, reduced both the induced permeability and MLC phosphorylation.

Despite the observed increase in RhoA activity in EA.hy926 cells, minimal to no increases in MLC phosphorylation were observed in response to thrombin. In addition, while the thrombin-induced reorganization of the actin cytoskeleton was observed to result in increased amounts of cortical stress fibres, formation of new stress fibres was not quantified for EA.hy926 cells or HUVECs. As well as stress fibre formation and actomyosin contraction, passive cell retraction as a result of cytoskeleton rearrangement and loss of cell-cell junctions have been reported to play a key role in the contractile response of endothelial cells up to 60 min (reviewed in Bogatcheva et al., 2001).
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Although passive cell retraction could explain the thrombin-induced cell contraction within the time-course under investigation, it is also possible that thrombin-induced phosphorylation of CAMK (Calcium/calmodulin-dependent kinase) contributes to cell contraction by inducing the phosphorylation of the actin-binding protein filamin responsible for three dimensional actin network formation (reviewed in Bogatcheva et al., 2002). Treatment of bovine pulmonary endothelial cells with CaM kinase II inhibitors or with a cell-permeable N-myristoylated synthetic filamin peptide (containing the COOH-terminal CaM kinase II phosphorylation site) attenuates thrombin-induced filamin phosphorylation, thrombin induced increases in permeability and transendothelial electrical resistance without affecting thrombin-induced MLC phosphorylation, thereby suggesting that CaM kinase II and filamin could be involved in the regulation of cytoskeletal reorganization in response to thrombin (Borbiev et al., 2001). Further investigation as to the mechanism by which thrombin induces cell contraction in EA.hy926 cells could therefore include its effect on the phosphorylation of CAMK and filamin. Whether the observed thrombin-induced contraction in HUVECs was mediated by myosin II was not determined. It would be interesting to determine the above mechanisms of cell contraction under the conditions investigated in HUVECs as well and compare those with EA.hy926 cells.

The role of the microtubule network in endothelial cell responses to thrombin has not been as extensively studied as that of the actin network. The observed decrease in the peripheral microtubule staining in response to thrombin induced as at early time-points of stimulation agrees with previous experiments carried out in pulmonary endothelial cells investigating the effect of thrombin on the microtubule network (Smurova et al., 2004). The decrease in peripheral staining was associated with reduced levels of acetylated tubulin suggesting localized destabilization of the microtubule filaments at cell periphery. Decreases in the levels of acetylated tubulin were observed to occur from the early time-points, starting at 2 min of thrombin stimulation, an observation that complements well with the immunofluorescence experiments on the microtubules. The involvement of G-proteins activated downstream the thrombin receptor (see Chapter 1.4.2) in inducing microtubule depolymerization was investigated by via inhibition of these proteins with
Pertussis toxin, which resulted in attenuation of thrombin-induced stress fibre formation and microtubule disassembly as well as via overexpression of their activated forms which resulted in increasing stress fibre density and complete microtubule disassembly (Smurova et al., 2004). As both, a decrease in microtubules at the cell periphery and slower formation of stress fibres in the cytoplasm were observed in EA.hy926 cells, the suggestion put forward by Smurova et al., (2004) that thrombin regulates the actin cytoskeleton of endothelial cells using local microtubule depolymerization at the cell edge, could be true for EA.hy926 cells. It would be interesting to study whether localized microtubule destabilization also occurred in HUVECs where thrombin-induced formation of stress fibres spanning the cell was not observed.

Additional experimental evidence regarding the involvement of microtubules in regulating cytokine-induced stress fibre formation was generated through investigating the effect of microtubule stabilizing agents in TNF-α-stimulated human pulmonary artery endothelial cells (Petrache et al., 2003). They reported that in addition to inhibiting cytokine-induced increases in actin stress fibres, microtubule stabilization with taxol inhibited the formation of intercellular gaps and restored the TNF-α-compromised VE-cadherin-based cell-cell junctions.

Accumulating evidence therefore implicates the microtubule network in the regulation of cytokine-induced cell contraction in terms of stress fibre content and junctional organization. As the requirement of the small Rho GTPase Cdc42 for restoration of cell-cell junctions following exposure to thrombin (Kooklis et al., 2004) has been reported, further investigation into the role of the microtubule network in thrombin-induced cell contraction should also involve assays related to its effects on Cdc42 activity.

3.7.2 The effect of endothelial cells to shear stress

As in the case of thrombin, the effect of shear stress on EA.hy926 cells has not yet been reported. From the published literature and observations of the current investigation it is evident that endothelial cell responses to shear stress depend not only on the cell type but also on the magnitude of applied shear stress. Interestingly, the results here revealed a
greater similarity between responses of EA.hy926 cells exposed to low shear stress and HUVECs exposed to high shear stress (although they exhibited different morphological characteristics) than between the same cell type (HUVECs) exposed to different shear stress magnitudes or the response of the different cell types (EA.hy926 and HUVECs) exposed to the same shear stress magnitude.

The differences in the effect on F-actin between the various conditions correlated well with the data obtained on the effect of shear stress on MLC phosphorylation. In contrast therefore to thrombin, shear stress-induced changes in the actin cytoskeleton appeared to be mediated, at least in part, via the phosphorylation of MLC.

Both EA.hy926 cells exposed to low and HUVECs exposed to high shear stress responded to flow without obvious alterations in stress fibre content or actin reorganization did not change the phosphorylated MLC levels. In contrast, HUVECs exposure to low shear stress induced the formation of stress fibres by 30 min and 60 min of application correlating with increased MLC. The timing of the events indicated that shear stress-induced MLC phosphorylation probably contributed (in part) to the observed changes.

Correlation between MLC phosphorylation and high shear stress (10 dyn/cm²) has been reported in bovine and human pulmonary artery endothelial cells which was associated with increased formation of stress fibres (Birukov et al., 2002). Interestingly, enhanced phosphorylation of MLC was also reported in porcine aortic endothelial cells exposed to 5 dyn/cm² shear stress (Watanabe et al., 1998). It therefore appears that diphosphorylated MLC (Ser19 and Thr18) has an important role in regulating the actin cytoskeleton in arteries in response to both high and intermediate shear stress magnitudes but also in veins exposed to low shear stress as indicated by the HUVEC response.

While the investigations carried out by both groups revealed the dependence of MLC phosphorylation on MLCK, Birukov et al., (2002) was also able to demonstrate that shear stress-induced cortical cytoskeletal re-arrangement was dependent upon the translocation
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of cortactin (an actin binding protein involved in the regulation of actin polymerization). Interestingly cortactin translocation was reported not to depend on MLCK activity. Instead, by overexpressing dominant negative Rac1, Birukov et al. (2002) revealed the dependence of shear stress-induced actin cytoskeletal reorientation and cortactin translocation on the activation of Rac1. Whether MLC phosphorylation alone was responsible for the shear stress-induced actin reorganization in HUVECs exposed to 1.6 dyn/cm² or whether additional factors such as cortactin translocation could have contributed to the observed effects should be the subject of further investigation.

The involvement of RhoA in regulating shear stress induced actin reorganization has been reported in both HUVEC and BAEC (Ridley, 2001; Tzima et al., 2001; Wojciak-Stothard and Ridley, 2003). Tzima et al. (2001) reported a transient inhibition of RhoA necessary for actin alignment in the direction of flow in BAEC (bovine aortic endothelial cells), which correlated with an initial decrease in stress fibre formation. A reduction in stress fibre content was observed after 60 min of low shear stress application on EA.hy926 cells and high shear stress application in HUVECs. It would therefore be interesting to investigate whether the decrease is the result of RhoA inhibition that would enable actin alignment to the direction of flow at a later time-point. Nevertheless, experiments investigating the effect of 3 dyn/cm² on RhoA activity in HUVEC revealed the transient activation of the protein, induced very early upon application of shear stress returning to basal levels with 10 min (Wojciak-Stothard and Ridley, 2003). The differences between the two response could be attributed to cell type, magnitude or differences in cell density (Tzima et al., 2003) used confluent BAECs while Wojciak-Stothard and Ridley, 2003 used subconfluent HUVECs). Activation of RhoA has been reported to be responsible for stress fibre formation, stress fibre alignment and cell elongation (Ridley, 2001). Cell alignment to the direction of flow was not observed in any of the conditions investigated but no published data exists indicating the occurrence of cell alignment as early as 60 min of shear stress application.
Investigating the effect of shearr stress on RhoA as well as Rac1 activity would provide valuable insight as to the mechanism by which the level of shear stress induced different responses in HUVEC.

In addition to actin reorganization and MLC phosphorylation, experiments carried out investigating the effect of shear stress on microtubule stability revealed the dependence of endothelial cell responses on the shear stress magnitude. As stress fibre formation has been reported to depend on the integrity of the microtubule network (Malek et al., 1996) it is not surprising that immunofluorescence experiments reveal an intact microtubule network after 60 min of high or low shear stress application in HUVEC. Yet, destabilization of tubulin occurred in HUVEC responding to 1.6 dyn/cm² shear stress. As was the case with actin, HUVEC response to high shear stress was similar to that of EA.hy926 cell response to low shear stress, characterized by no obvious changes in microtubule stability.

The fact that the microtubule network integrity was maintained implies that the decrease in the amount of acetylated tubulin was probably not the result of a direct disruption of the microtubule network. Decreased stability could enable the microtubule cytoskeleton to undergo dynamic reorganization in response to flow. Nevertheless, no remodelling including re-orientation of the microtubule organizing centre (MTOC) was evident at least not within the 1 hour time-course. Re-orientation of the MTOC next to the nucleus in the direction of flow has been reported in BAEC at later time points (Tzima et al., 2003) and is considered an important determinant of polarized movement (Malech et al., 1977; Koonce et al., 1984) regulated by the small Rho GTPase Cdc42 (Tzima et al., 2003).

Thus, the response of endothelial cells to shear stress was observed to be not only cell type specific but also magnitude-dependent, with greater similarities shared between EA.hy926 cells exposed to low shear stress and HUVECs exposed to high shear stress. Nevertheless, these response similarities were mostly based on negative results indicating that the two conditions could in reality signal through entirely unrelated pathways.
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including different receptors. In fact, the different morphological changes associated with the EA.hy926 and HUVEC response to low and high shear stress respectively, is consistent with this possibility. In contrast, no particular dependence on cell type was observed in endothelial responses to thrombin, yet a more extensive comparison between the HUVEC and EA.hy926 cells could reveal that this is not so.

As the activity of integrins was not investigated here, it is not possible to determine whether the observed changes are occurring downstream activation of integrins. In addition to integrin receptors shear stress has been reported to activate many other receptors such as PECAM-1, calcium channels or VEGFR2 (see Chapter 1.4.1). It is therefore possible that different receptors or different combinations of receptors are activated by flow depending either on cell type, shear stress magnitude or both.

Also of importance is the comparison between the thrombin and shear stress; while actin reorganization and stress fibre formation were observed to be a common response of HUVECs exposed to shear stress and thrombin-stimulated EA.h926 cells, different mechanisms appear to be responsible in regulating each of these changes in the two responses.
4 The effect of thrombin and shear stress on focal adhesion proteins

4.1 Introduction

The precise spatial localization of the mechanotransduction sites in endothelial cells is not yet known (see chapter 1.4.1). It is clear though that the force is likely to be transmitted to several locations rich in enzymes involved in signal transduction (Davies, 1995). Focal adhesion sites fulfill these requirements and have therefore been considered good candidates for force transmission, for example in response to shear stress. Integrins localize, in part, at focal adhesion sites and have been reported to be activated both in response to shear stress and thrombin (see chapter 1.5).

The mechanism by which thrombin activates integrin signalling is not yet fully understood: it is hypothesized that thrombin induces activation of integrins via an indirect interaction between the thrombin receptor PAR-1 and integrins (Chung et al., 1999). The activity of some focal adhesion proteins has been reported to be affected in response to thrombin and shear stress and they also activate signal transduction pathways leading to the regulation of Rho and Rac GTPases (see Chapter 1.6 and 1.7).

Amongst the proteins that have been reported to be phosphorylated in response to both thrombin and shear stress are FAK, Src and paxillin (see Chapter 1.6). Although the two stimuli appear to activate similar endothelial cell proteins, their effect on endothelial cell morphology was quite different (see Chapter 3). This chapter investigates the effects of thrombin and shear stress on the tyrosine phosphorylation and localization of FAK, Src and paxillin in EA.hy926 cells (which has not been reported to date) in order to provide insight into how the two stimuli induce these effects. Cell type specificity was investigated by comparing EA.hy926 cell response to low shear stress and the BAEC response to high shear stress to that of HUVEC (vein vs vein; artery vs vein). The effect of shear stress on FAK, Src and paxillin has been previously investigated in BAECs. By comparing the HUVEC response to different magnitudes of shear stress the dependence of endothelial cell responses to flow on shear stress magnitude was also investigated.
4.2 The effect of thrombin and shear stress on tyrosine phosphorylation of FAK

Upon integrin activation, the non-receptor tyrosine kinase, FAK, has been reported to undergo auto-phosphorylation at the tyrosine residue Y397 thereby allowing the protein to recruit the tyrosine kinase Src (Short, 1998), which could be responsible for the phosphorylation of other FAK residues including Y576 (Comillon et al., 2003). The effect of thrombin and low shear stress on the phosphorylation of the specific tyrosine residues was investigated in EA.hy926 cells: confluent monolayers of EA.hy926 cells were incubated with thrombin or exposed to low shear stress and the lysates were used to detect any changes in the phosphorylation of FAK at residue sites Y397 and Y576. For comparison between different cell types and conditions, the effect of shear stress on FAK phosphorylation was also investigated in HUVECs exposed to low or high shear stress levels and in BAECs exposed to high shear stress. The effect of high shear stress on EA.hy926 cells was not analyzed as a significant amount cell detachment occurred as a result of high shear stress levels (see chapter 3).

4.2.1 Thrombin induces FAK phosphorylation in EA.hy926

Thrombin induced statistically significant increases in the phosphorylation of both Y397 and Y576 FAK residues, at 60 min and 10 min of stimulation respectively (Figure 4.1a). Quantifications of pY576 FAK levels revealed that a slight increase (~10%) was induced by 10 min of thrombin stimulation reaching a peak (~20%) by 60 min. These increases were small thereby debating the significance of the quantified changes. An increase of pY397 FAK (Figure 4.1a) was observed by 10 min of thrombin stimulation and maintained throughout the time-course. The fold-difference, in phosphorylation at the 5 and 30 min time-points and control varied between experiments and were therefore not statistically significant (Figure 4.1b).
Figure 4.1: The effect of thrombin on FAK phosphorylation
Confluent EA.hy926 cells were exposed to 2 U/ml of thrombin for the indicated times. 

a) After treatment, cells were lysed in sample buffer, electrophoresed on a 4-12 % SDS-

PA gel and probed with phosphospecific antibodies to a pY397-FAK b pY576-FAK. 

FAK and ERK were used as loading controls b) shows pFAK levels of treated cells 

relative to pFAK levels of control cells +/- s.e.m. for three independent experiments. 

ERK was used as the loading control for quantifications. Statistical significance was 

determined using ANOVA and Student’s t-test (* p<0.05).
4.2.2 Low shear stress induces FAK phosphorylation in EA.hy926 cells but not HUVECs

Low shear stress induced a significant increase in the phosphorylation of both Y397 and Y576 FAK residues in EA.hy926 cells, and minor insignificant decreases in the phosphorylation of both in HUVECs (Figure 4.2b).

In EA.hy926 cells phosphorylation of both residues was induced after 2 min of shear stress application. Even though both changes proved statistically significant, the fold increase was ~60% higher for Y397 than Y576 (1.9 and 1.25 fold, respectively). The two residues followed similar phosphorylation trends with peak levels (2.5 and 1.5 fold for Y397 and Y576 respectively) reached after 5 min of shear stress application followed by a steady decline. Nevertheless, phosphorylation of Y397 was higher than control throughout the time-course, while by 60 min of shear stress application phosphorylation of Y576 had almost reached basal levels.

In contrast to EA.hy926 cells, low shear stress did not induce the phosphorylation of either of the two FAK residues in HUVECs. If anything dephosphorylation (<20%) of the two residues occurred after 5, 10 and 60 min of shear stress application with a very slight increase at the 30 min time-point, reaching phosphorylation levels of the control. While all of the changes observed were minor and did not prove to be statistically significant, the trends were similar between experiments, suggesting that what appears to be “no change” could, with additional experiments, prove to be a small but consistent dephosphorylation of the residues in response to low shear stress.

4.2.3 High shear stress induces FAK phosphorylation in HUVECs and in BAECs

High shear stress induced an increase in the phosphorylation of both Y397 and Y576 residues in the two cell types, HUVEC and BAEC (Figure 4.3). With the exception of pY397 in HUVEC, the majority of the changes were statistically significant. All trends were consistent between experiments including those of the Y397 residue in HUVECs.
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Figure 4.2: The effect of low shear stress on FAK phosphorylation

Confluent EA.hy926 cell and HUVEC monolayers were exposed to 1.6 dyn/cm² shear stress for the time-periods indicated. a) After treatment, cells were lysed in sample buffer, electrophoresed on a 4-12 % SDS-PA gel and probed with antibodies to pY397-FAK and pY576-FAK. b) and c) show pFAK levels of treated EA.hy926 cells and HUVECs respectively, relative to pFAK levels of control cells +/- s.e.m for three independent experiments. FAK and ERK were used as loading controls for quantifications of EA.hy926 cells and HUVECs respectively. Statistical significance was determined using ANOVA and Student’s t-test (* p<0.05).
The effect of thrombin and shear stress on focal adhesion proteins

Figure 4.3: The effect of high shear stress on FAK phosphorylation

Confluent HUVEC and BAEC monolayers were exposed to 12 dyn/cm² of shear stress for the time-periods indicated. a) After treatment, cells were lysed in sample buffer, electrophoresed on a 4-12% SDS-PA gel and probed with antibodies to pY397-FAK and pY576-FAK. b) and c) show pFAK levels of treated HUVEC and BAEC respectively, relative to pFAK levels of control cells +/- s.e.m. for three independent experiments. ERK and FAK were used as loading controls for the quantification of HUVEC and BAEC respectively. Statistical significance was determined using ANOVA and Student’s t-test (* p<0.05).
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Shear stress induced phosphorylation of both residues after 2 min of application in both cell types, with levels remaining higher than basal throughout the time-course. The levels of pY397 only increased by a maximum of 1.5 fold, and once they had peaked (2 min and 5 min in HUVEC and BAEC respectively) remained approximately at the same levels throughout the time course. The levels of pY576 on the other hand, were observed to peak (around 2 fold increase) after 5 and 10 min of shear stress application in HUVEC and BAEC respectively followed by a steady decline. Interestingly, the peak of pY576 in each cell type occurred immediately after the time-point at which pY397 levels were observed to peak (2 min in HUVEC and 5 min in BAEC).

Both thrombin and shear stress induced changes in the phosphorylation of the two FAK residues. With the exception of HUVECs exposed to low shear stress, where slight dephosphorylation of Y397 occurred, the phosphorylation levels of both residues increased to varying degrees in response to stimulation.

Cell type specificity in high shear stress-induced changes was not observed to occur; other than perhaps differences in the folds of phosphorylation levels, cells derived from veins (HUVEC) and arteries (BAEC) exhibited similar responses in terms of FAK phosphorylation. Yet, the response of EA.hy926 cells and HUVECs to low shear stress differed greatly, thereby showing that shear stress responses are cell-type dependent. The response of the same cell type (HUVEC) was markedly differed at low versus high shear stress magnitudes, indicating that the shear stress level could determine the behaviour of the endothelial monolayer.

Interestingly Y576-FAK followed a similar pattern of phosphorylation as that of Y397 residue in response to shear stress stimulation, indicating that the phosphorylation status of the two residues is linked. When the phosphorylation of the two residues was not simultaneous (HUVECs and BAECs, 12 dyn/cm²), the increase of pY397 preceded that of pY576, suggesting that the phosphorylation of Y576 is dependent upon FAK auto-phosphorylation, as would be expected from the model proposed by Short et al. (1998) (see Figure 1.7). Whereas like shear stress, thrombin induced the phosphorylation of
Y397 in EA.hy926 cells, the response was later (10 min as opposed to 2 min) and the peak levels were less (1.5-fold as opposed to ~2-fold). Differences in timing and levels could explain the small yet consistent increases in pY576 in response to thrombin, are higher compared to the high changes in FAK auto-phosphorylation.

4.3 The effect of thrombin and shear stress on Src phosphorylation
As mentioned before, integrin-induced FAK auto-phosphorylation at Y397 has been reported to induce the recruitment of Src, which could then be responsible for the phosphorylation of the Y576 FAK residue (Short et al., 1998). Src kinase activity is induced by phosphorylation of its tyrosine residue Y416 (reviewed in Petrone and Sap, 2000). In order to determine whether the changes in the phosphorylation of Y576 FAK could be the result of the action of Src kinase, the effect of thrombin and shear stress on the phosphorylation of Y416 Src was investigated. Confluent monolayers of EA.hy926 were incubated with thrombin or exposed to low shear stress and tested for changes in the phosphorylation of pY416-Src. The effect of shear stress on pY416-Src was also investigated under high shear stress conditions in BAECs.

4.3.1 Both thrombin and shear stress induce the phosphorylation of Y416-Src
Increases in pY416-Src occurred in EA.hy926 cells in response to both thrombin and low shear stress as well as in BAECs exposed to high shear stress (Figure 4.4). Thrombin induced a small increase (~10%) in Y416-Src phosphorylation after 2 min of stimulation, a slightly higher increase at 30 min (~24%), which declined after 60 min (~10%).

An increase in pY416-Src was also observed in EA.hy926 cells exposed to low shear stress, yet with greater fold differences and with a slightly different timing. The initial small increase (~20%) in pY416-Src occurred after 2 min of shear stress application followed by a peak (~55% increase) at 10 min followed by a 10% reduction in pY416-Src levels (~45%) at the 30 and 60 min time-points. Phosphorylation of Y416 under high shear stress followed a similar pattern in BAEC peaking at 10 min with a 2.5-fold increase. As with low shear stress in EA.hy926 cells, phosphorylation remained at higher than basal levels with ~50% increase characterizing the 60 min time-point.
The effect of thrombin and shear stress on focal adhesion proteins

Figure 4.4: The effect of thrombin and shear stress on the phosphorylation of Src
Confluent EA.hy926 cells were exposed to a) 2 U/ml of thrombin or b) 1.6 dyn/cm² shear stress and c) confluent BEACs were exposed to 12 dyn/cm² shear stress. After treatment cells were lysed in sample buffer, electrophoresed on a 4-12 % SDS-PA gel and probed with pY416-Src. Total Src and ERK were used as loading controls. d) shows the pSrc levels of treated cells relative to pY416Src levels of control cells +/- s.e.m. for three independent experiments per condition. ERK and Src were used as loading controls for the quantifications of EA.hy926 cells and BAECs respectively. Statistical significance was determined using ANOVA and Student’s t-test (* p<0.05).
An increase in Src phosphorylation, indicative of activation, occurred in endothelial cells in response to both thrombin and shear stress. Under all three conditions investigated, initial increases in Src activity occurred either simultaneously or at the time-point directly preceding induction of pY576-FAK. Interestingly, fold increases in pY416-Src correlated with fold increases in pY576-FAK: the smallest increase in pY416-Src occurred in thrombin-stimulated EA.hy926 cells where levels of pY576-FAK did not exceed a 20% increase while the biggest increase (2.5-fold) occurred in BAECs exposed to high shear stress and was followed by a two-fold increase in the levels of pY576-FAK. Although the above observations do not prove that Y576-FAK phosphorylation results is caused by Src kinase, they provide evidence that Src activation correlates with Y576-FAK phosphorylation. While shear stress induced a transient peak in pY416-Src, thrombin did not, as the shear stress response appears to be more rapid compared to thrombin. It is possible that pY416-Src follows the same pattern in thrombin-stimulated EA.hy926 cells as in response to shear stress and that decreases in pY416-Src levels occur at later time-points later than 60 min.

4.4 The effect of thrombin and shear stress on tyrosine phosphorylation of paxillin

Increases in the phosphorylation of paxillin at Y31 and Y118 residues have been reported in response to thrombin and shear stress (Hollinstat et al., 2006; Mattiussi et al., 2006). It has also been suggested that Y118 phosphorylation is induced by either FAK or Src (Ischibe et al., 2003) after the recruitment of paxillin to the FAK-Src complex (Plopper and Ingber 1993). Phosphorylation of Y31-paxillin could be induced by FAK (Guo et al., 2006). In addition, the phosphorylation of the two paxillin residues has been implicated in the regulation of Rho and probably Rac GTPase (Tsoubourchi et al., 2002, chapter 1.2.8), whose activity was observed to increase in EA.hy926 cells response to thrombin stimulation (chapter 3.4.1).
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Figure 4.5: The effect of thrombin on paxillin phosphorylation
Confluent EA.hy926 cells were exposed to 2 U/ml of thrombin. a) After treatment, cells were lysed in sample buffer, electrophoresed on a 4-12 % SDS-PAGE gel and probed with phosphospecific antibodies to pY118-paxillin and pY31-paxillin. Paxillin and ERK were used as loading controls. b) shows pPaxillin levels of treated cells relative to control cells +/- s.e.m. for three independent experiments. ERK was used as a loading control for quantifications. Statistical significance was determined using ANOVA and Student’s t-test (* p<0.05).
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Figure 4.6.: The effect of low shear stress on paxillin phosphorylation
Confluent EA.hy926 cell and HUVEC monolayers were exposed to 1.6 dyn/cm² of shear stress for the time-periods indicated. a) After treatment, cells were lysed in sample buffer, electrophoresed on a 4-12 % SDS-PAGE gel and probed with antibodies to pY118-paxillin and pY31-Paxillin b) and c) show pPaxillin levels relative to pPaxillin levels of control cells +/- s.e.m for three independent experiments. Paxillin and ERK were used as loading controls for the quantifications of EA.hy926 cells and HUVECs respectively. Statistical significance was determined using ANOVA and Student’s t-test (* p<0.05). + indicates statistically significant change between the 5 and 10 min time-points.
As before, EA.hy926 cells were exposed to thrombin or low shear stress, HUVECs were exposed to low or high shear stress while BAECs were exposed to high shear stress. By comparing the effect of the different conditions on the phosphorylation of Y31- and Y118-paxillin with Y397-FAK and Y416-Src it could be determined whether paxillin phosphorylation correlated with Src activation and/or FAK phosphorylation and whether the two paxillin residues could be implicated in the thrombin-induced transient increase in RhoA activity (chapter 3.4.1).

4.4.1 Thrombin induced increases in pY118 and decreases in pY31-paxillin levels
Slight changes in the levels of pY31 and pY118-paxillin in response to thrombin were observed to be induced after 10 min of stimulation: the levels of pY118-paxillin increased with time, whereas pY31-paxillin decreased (Figure 4.5). By 60 min phosphorylated levels of the two residues had changed by the same amount (~40%) in opposite directions. These were the maximum changes in the levels of pY118 and pY31-paxillin in thrombin-stimulated EA.hy926 cells. The pattern of changes was consistent between three independent experiments. Nevertheless, variations in the fold-increase/decrease between the experiments meant that the differences were not statistically significant (with the exception of pY118-paxillin at the last time-point).

4.4.2 Low shear stress increases pY118 and decreases pY31-paxillin levels
In order to compare the thrombin response with shear stress in EA.hy926 cells, the effect of 1.6 dyn/cm² shear stress application on paxillin phosphorylation was investigated (Figure 4.6). After exposure to low shear stress the pY31- and pY118-paxillin followed similar changes in phosphorylation to thrombin-stimulated cells: whereas levels of pY118-paxillin increased in response to shear stress a reduction in pY31-paxillin levels occurred. Differences between thrombin and low shear stress included an earlier induction of the observed changes. Increased paxillin phosphorylation (~50%) at Y118-paxillin and dephosphorylation (~30%), at Y31- paxillin, was detected as early as 2 min of shear stress application. Similar to thrombin stimulation, phosphorylation levels remained higher and lower than basal for Y118- and Y31- paxillin respectively throughout the 60 min time-course. In contrast to thrombin, a certain degree of
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dephosphorylation was observed to occur at the Y118 residue after 10 min exposure of EA.hy926 cells to shear stress. Although only the changes in pY31-paxillin proved statistically significant, the pattern of phosphorylation for Y118-paxillin was consistent between three independent experiments, but fold-increases between experiments were quite different and the changes therefore not statistically significant.

The effect of 1.6 dyn/cm² shear stress application on paxillin phosphorylation was investigated in HUVECs, in order to determine whether similar responses occur in the two cell types (Figure 4.6). Both responded almost identically, inducing the phosphorylation of Y118-paxillin and dephosphorylation of Y31-paxillin. As experiments carried out in HUVECs lacked the 2 min time-point, it was not possible to observe whether the changes were induced at the same time. After 5 min of shear stress application, Y118-paxillin phosphorylation was observed to increase by a small amount (~20%) while a reduction of the same amount (~20%) was observed for pY31-paxillin. Both these changes, were small but statistically significant.

The small dephosphorylation of pY118-paxillin observed in EA.hy926 cells (Figure 4.4b) also occurred in HUVECs, although increased phosphorylation again reaching a maximum after 60 min of shear stress application (1.5 fold increase). Neither the decrease in pY118-paxillin levels after 10 min of shear stress nor the increase at the 60 min time-point were statistically significant compared to basal levels. Yet, the difference in pY118-paxillin levels between the 5 and 10 min time-points proved statistically significant. As in EA.hy926 cells levels of pY31-paxillin remained lower than those of the control throughout the time-course.
4.4.3 High shear stress increases pY118 and decreases pY31-paxillin levels

In order to compare the effect of different shear stress magnitudes on the same cell type, HUVECs were exposed to 12 dyn/cm² shear stress and the effect on the phosphorylation status of Y118 and Y31-paxillin was investigated (Figure 4.7). Similar to low shear stress, high magnitudes were observed to induce the phosphorylation of Y118-paxillin. As opposed to the HUVEC and EA.hy926 response to low magnitude shear stress, dephosphorylation did not follow the initial increase in HUVECs exposed to 12 dyn/cm². Instead, pY118-paxillin levels were observed to undergo a small statistically significant increase proportional to time, reaching a peak at 60 min (~20% increase compared to basal levels). In contrast to Y118-paxillin, dephosphorylation of Y31 was observed in HUVECs exposed to high shear stress, reaching a maximum of around 30% reduction after 30 to 60 min of shear stress. The changes in paxillin phosphorylation occurred early in shear stress-stimulated HUVEC irrespective of stress magnitude and were strongest after 60 min of application.

The effect of 12 dyn/cm² on paxillin phosphorylation was also investigated in BAECs (Figure 4.7) allowing for comparisons between cells derived from veins (HUVEC) and arteries (BAEC) in response to flow. An increase in pY118-paxillin observed in BAEC after 2 min of shear stress application and levels were maintained at higher than control throughout the time-course. In contrast to the HUVEC response to high shear stress application but similar to low shear stress application in both HUVECs and EA.hy926 cells, a slight dephosphorylation of pY118-paxillin was observed after 10 min and a subsequent increase at 30 and 60 min of shear stress application. Observations were consistent between three independent experiments. A small yet significant decrease in the phosphorylation of Y31-paxillin was observed when BAECs exposed to 2 min of 12 dyn/cm² shear stress (Figure 4.7c). The 30% reduction in phosphorylation achieved by 30 min was sustained after 60 min and proved statistically significant for both time-points. At the time-points in between (5 and 10 min of shear stress), pY118-paxillin was similar to control levels for two out of the three experiments while for the third experiment an approximately 30% reduction was achieved by the 10 min time-point and was maintained throughout.
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Figure 4.7: The effect of high shear stress on paxillin phosphorylation
Confluent HUVEC and BAEC monolayers were exposed to 12 dyn/cm² of shear stress for the time-periods indicated. a) After treatment, cells were lysed in sample buffer electrophoresed on a 4-12 % SDS-PAGE gel and probed with antibodies to pY118-paxillin and pY31-paxillin. b) and c) show pPaxillin levels of treated cells relative to control cells +/- s.e.m for three independent experiments. ERK and paxillin were used as loading controls for the quantifications. Statistical significance was determined using ANOVA and Student’s t-test (* p<0.05).
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In summary, in response to thrombin and shear stress, the two tyrosine residues of paxillin followed two opposite phosphorylation profiles irrespective of cell type or shear stress magnitude. The timing of the events differed between experiments, with shear stress inducing the changes more rapidly compared to thrombin.

In thrombin-stimulated EA.hy926 cells (Figure 4.8a), changes in paxillin phosphorylation were induced around the same time as Y397-FAK and Y576-FAK phosphorylation (10 min) and after the increase in active Src (2 min). Simultaneous induction of pY397-FAK and pY576-FAK with pY118-paxillin phosphorylation and pY31-paxillin dephosphorylation (2 min) also occurred in EA.hy926 cells exposed to 1.6 dyn/cm² shear stress (Figure 4.08b). Under shear stress, the observed changes also correlated with the induction of pY416-Src. EA.hy926 cells therefore, responded in similar ways to thrombin and shear stress but the response to flow was more rapid and of a greater magnitude. The timing of the events showed that both Y118-paxillin phosphorylation and Y31-paxillin de-phosphorylation could be dependent upon the phosphorylation of the two focal adhesion proteins FAK and Src.

Whereas in HUVECs, shear stress (1.6 dyn/cm²) did not induce FAK phosphorylation (chapter 4.2.2), the pY118- and pY31- paxillin changes observed in EA.hy926 cells exposed to the same magnitude of flow also occurred in HUVECs (Figure 4.8c). It is therefore possible that paxillin phosphorylation and dephosphorylation of Y118 and Y31 residues respectively, is not (entirely) dependent on FAK phosphorylation. The HUVEC response to 12 dyn/cm² (Figure 4.8d) was quite different to its response to low shear stress and more similar to the response of EA.hy926 cells exposed to 1.6 dyn/cm²: under high shear stress, changes in paxillin phosphorylation coincided with changes in FAK phosphorylation as was the case with thrombin and shear stress-stimulated EA.hy926 cells. When high shear stress was applied to BAECs (Figure 4.08e), pY576-FAK and pY416-Src increased after the induction of pY397-FAK and changes in paxillin phosphorylation (2 min), implying that factors other than increased FAK and/or Src phosphorylation contribute to the regulation of pY118-paxillin phosphorylation.
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**Thrombin (EA.hy926)**

- **a**
  - Graph showing the ratio of phosphorylated levels (treated/control) over time (min) for pY397-FAK, pY576-FAK, pY416-Src, pY118-paxillin, and pY31-paxillin.

- **b**
  - Graph showing the ratio of phosphorylated levels (treated/control) over time (min) for pY397-FAK, pY576-FAK, pY118-paxillin, and pY31-paxillin.

- **c**
  - Graph showing the ratio of phosphorylated levels (treated/control) over time (min) for pY397-FAK, pY576-FAK, pY118-paxillin, and pY31-paxillin.
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Figure 4.8: Summary of FAK, Src and paxillin phosphorylation changes
Quantifications of three independent experiments for the following conditions
a) thrombin stimulated (2 U/ml) EA.hy926 cells for the time-periods indicated
b) shear stress (1.6 dyn/cm²) applied to EA.hy926 cells for the time-periods indicated
c) shear stress (1.6 dyn/cm²) applied to HUVECs for the time-periods indicated
d) shear stress (12 dyn/cm²) applied to HUVECs for the time-periods indicated
e) shear stress (12 dyn/cm²) applied to BAECs for the time-periods indicated
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Interestingly, under all conditions investigated and in all cell types, the opposite changes in phosphorylation of the two paxillin residues occurred simultaneously, indicating that the phosphorylation of Y118-paxillin could be dependent on or facilitated by the dephosphorylation of Y31-paxillin and vice versa or that they are both regulated by the same upstream event. Comparisons between phosphorylation trends of all proteins investigated, within the 0 to 10 min range, provided no indication of why the slight dephosphorylation of Y118-paxillin, consistently observed when EA.hy926 cells or HUVECs were exposed to 1.6 dyn/cm² shear stress for 10 min, occurred.

4.5 Effect of thrombin and shear stress on protein localization and focal adhesions

Phosphorylated paxillin, Src and FAK have all been reported to localize in focal adhesions (see Chapter 1.6.6). Immunofluorescence experiments were therefore carried out in confluent EA.hy926 cells and HUVECs exposed to either thrombin or shear stress in order to investigate their effect on pY118-paxillin, pY397-FAK, pY576-FAK and pY416-Src localization.

4.5.1 The effect of thrombin on FAK and Src localization

Immunofluorescence experiments revealed the localization of pY397-FAK at focal adhesions under unstimulated and thrombin-stimulated conditions (Figure 4.9a). Some cytoplasmic staining was also observed. Incubation of EA.hy926 cells with thrombin did not have an obvious effect on the number or the size of focal adhesions.

Preliminary immunofluorescence experiments with the phospho-specific antibody for Y576-FAK (Figure 4.9c), showed a more diffuse localization of pY576-FAK compared to pY397-FAK. The punctate staining observed resembled a vesicular staining pattern. Nevertheless, as immunofluorescence with antibodies staining for vesicles was not carried out, the specificity of the antibody for vesicular staining could not be confirmed. Reservations as to the specificity of pY576-FAK antibody were reinforced as in this particular experiment, staining with the secondary antibody alone resulted in somewhat similar but fainter staining of the cells (not shown).
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(a) F-actin  pY397-FAK  pY397-FAK  Merge

(b) 0 min  10 min  60 min
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Figure 4.9: Localization of pY397-FAK, pY576-FAK and pY416 Src
EA.hy926 cells were stimulated with 2 U/ml of thrombin. After treatment cells were
fixed and stained for a) F-actin and pY397-FAK. Representative of three experiments.
The third column shows a magnification of the area indicated in the pY397-FAK
column to its left b) VE-cadherin and pY416-Src. Representative of three experiments;
bottom panel shows a magnification of the area indicated in the upper panel c) F-actin
and pY576-FAK. Taken from one experiment. Bar=20 μm; red and grey arrows point to
focal adhesions
Like pY397-FAK, pY416-Src was observed to localize to focal adhesions under basal and thrombin-stimulated conditions (Figure 4.9b). However, in contrast to pY397-FAK, pY416-Src staining of thrombin-stimulated EA.hy926 cells revealed thicker and more elongated focal adhesions compared to unstimulated cells, suggesting increasing translocation of activated Src to the sites of adhesion. Nevertheless, the absence of F-actin staining in parallel to pY416-Src as well as lack of quantification of focal adhesion numbers and size meant that no firm conclusions can be drawn with respect to the localization and translocation of the focal adhesion protein to these sites of adhesion.

4.5.2 The effect of thrombin on paxillin localization
Under basal conditions, paxillin was localized predominantly to the cytoplasm as indicated by the perinuclear staining (Figure 4.10a). After 30 min of thrombin stimulation, paxillin was observed to translocate to the end of stress fibres indicating the formation of new focal adhesions or recruitment of paxillin to already formed focal adhesions. In contrast, under basal conditions pY118-paxillin localized specifically at focal adhesions, both in EA.hy926 cells (Figure 4.10b) and in HUVECs (Figure 4.11), indicating localized phosphorylation and enrichment of Y118-paxillin at the sites of adhesion. After 30 min of shear stress stimulation, thicker and perhaps more elongated focal adhesions were observed in HUVECs (and possibly EA.hy926 cells), indicating an increase in the level of pY118-paxillin to these sites. In response to thrombin, focal adhesions were mainly localized at the cell periphery.

4.5.3 The effect of shear stress on paxillin localization
Application of 1.6 dyn/cm² shear stress on EA.hy926 cells (Figure 4.12) did not have an obvious effect on the localization of pY118-paxillin. The phosphorylated protein was localized in part to focal adhesions, whose size and number appeared to remain more or less constant at 10 and 30 min of shear stress application. With increasing spreading and a decrease in the number of stress fibres, less focal adhesions were observed after a 12-hour exposure of EA.hy926 cells to shear stress. In contrast, increasing levels of pY118-paxillin at focal adhesions were observed in HUVECs exposed to 1.6 dyn/cm² shear stress (Figure 4.13) indicated by the more elongated (and probably greater numbers) focal
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adhesions at the 30 and 60 min time-points compared to the 10 min time-point and untreated control. As in the case of pY416-Src though, quantifications of focal adhesion numbers and size should be carried out to allow accurate comparison between conditions.

The HUVEC response to high shear stress (Figure 4.14) resembled the EA.hy926 cell response to low shear stress, as an increase in the number of focal adhesions or translocation of pY118-paxillin to these sites did not occur. Interestingly from the pY118-paxillin staining it was observed that in HUVECs focal adhesions are more elongated than in EA.hy926 cells. The lower level of cytoplasmic pY118-paxillin staining (and probably pY416-Src.), suggested the localized phosphorylation of the protein(s) at focal adhesions but based on the cytoplasmic staining exhibited by the pY397-FAK antibody, the same conclusion could not be drawn for pY397-FAK.

Immunofluorescence experiments thus revealed the specific localization of pY397-FAK, pY118-paxillin and pY416-Src to focal adhesions under basal and stimulated conditions.

Despite the lack of quantification, certain conclusions can be drawn based on the immunofluorescence experiments: the formation of thicker or more elongated focal adhesions in response to thrombin, observed through pY118-paxillin and pY416-Src staining, suggesting probable translocation of more paxillin and Src to these sites. In the case of pY118-paxillin, maturation was not observed until after 30 min of thrombin stimulation. Thicker and elongated focal adhesions were revealed by the pY416-Src antibody at the 10 min time-point compared to control, thereby indicating that pY416-Src translocation to focal adhesions precedes that of pY118-paxillin.

In contrast to thrombin-stimulated EA.hy926 cells, elongation of focal adhesions was not observed in response to shear stress, indicating that although pY118-paxillin at sites of adhesion, shear stress did not induce the translocation of more pY118-paxillin to focal adhesions. In contrast the HUVEC response to shear stress in terms of pY118-paxillin localization resembled that of thrombin-stimulated HUVECs. Yet in the thrombin response, elongation of focal adhesions was only observed after 60 min of stimulation.
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whereas under shear stress elongated focal adhesions were induced after 30 min and maintained until the 60 min time-point. As well as cell-type specificity the response of pY118-paxillin appeared to depend on the magnitude of shear stress applied, as when exposed to high shear stress, no obvious effect was exerted on the localization of the phosphorylated protein in HUVECs.

Unfortunately, due to insufficient cell numbers, immunofluorescence experiments were not carried out in BAECs exposed to high shear stress. Hence, comparisons between the response of BAECs and other cell types could not be made. In addition, the phospho-specific antibodies for Y416-Src, Y397-FAK (and Y576-FAK) did not give a detectable signal on the plastic on which shear stress experiments were carried out, while staining with pY31-paxillin gave non-specific staining on both glass (thrombin experiments) and plastic (shear stress experiments) so that the localization of the pY31-paxillin could not be determined.
Figure 4.10: Thrombin induced localized phosphorylation of Y118-paxillin in EA.hy926 cells
Confluent EA.hy926 cells were stimulated with 2 U/ml of thrombin for the time-periods indicated and were stained for a) F-actin and total paxillin or b) F-actin and pY118-paxillin. Right column shows the magnification of the area indicated in the pY118-paxillin column on its left. Representative of at least three experiments. Bar=20 μm, red arrows point to focal adhesions.
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Figure 4.11.: pY118-paxillin localization to focal adhesions in HUVECs
Confluent HUVECs were exposed to 2 U/ml of thrombin for the time periods indicated and were stained for F-actin and pY118-paxillin. The third column Representative of three experiments. Bar=20 μm. Red and grey arrows point to focal adhesions
Confluent EA.hy926 cells were exposed to 1.6 dyn/cm² of shear stress for the time periods indicated, and stained for F-actin and pY118-paxillin. Representative of 3 experiments except the 12 hour time-point, which is taken from one experiment. The third column shows the magnification of the area indicated in the pY118-paxillin column on its left. Red and grey arrows point towards focal adhesions. Black arrow (bottom left) indicates flow direction. Bar=20 μm. Uneven shading across images is the consequence of uneven antibody distribution on the shear stress chamber surface due to the nature of the chambers.
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Figure 4.13: Low shear stress and pY118-paxillin localization to focal adhesions
Confluent HUVECs were exposed to 1.6 dyn/cm² of shear stress for the time periods indicated, then stained for F-actin and pY118-paxillin. Representative of at least three experiments. Grey arrows point to focal adhesions. The black arrow indicates flow direction. Bar=20 μm. Uneven shading across images is the consequence of uneven antibody distribution on the shear stress chamber surface due to the nature of the chambers.

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Figure 4.14: High shear stress and pY118-paxillin localization to focal adhesions
Confluent HUVECs were exposed to 12 dyn/cm² of shear stress for the time period indicated, then stained for F-actin and pY118-paxillin. The third column shows a magnification of the area indicated in the pY118-paxillin column on its left. Representative of three experiments. Red and grey arrows point to focal adhesions. The black arrow (bottom left) indicates flow direction. Bar=20 µm. Uneven shading across some images is the consequence of uneven antibody distribution on the shear stress chamber surface due to the nature of the chambers.
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4.6 Discussion

Although the effect of thrombin and shear stress on protein tyrosine phosphorylation has been reported in a variety of cell types including HUVECs and BAECs, no published data exist for EA.hy926 cells. The aim of this work was to attempt to deduce whether a specific pattern exists in terms of the phosphorylation status of the different focal adhesion proteins and/or at different residues of the same protein. Here I will focus on the effect on the phosphorylation status of focal adhesion proteins in terms of timing and not fold activations which varied between experiments (Figure 4.8).

4.6.1 Thrombin-induced FAK phosphorylation

Phosphorylation of FAK has been reported in response to various concentrations of thrombin in both vein- and artery-derived endothelial cells (HUVEC and PAEC: pulmonary artery endothelial cells). An overall increase in tyrosine phosphorylation of FAK in thrombin-stimulated PAEC was observed as early as 2 to 5 min after stimulation (Schaphost et al., 1997).

Thrombin-induced phosphorylation of specific FAK residues was described in PAEC by Hollinstat et al. (2005) and Shikata et al. (2003), who reported increases in pY397-FAK and pY576-FAK in response to thrombin occurring at later time-points to total FAK phosphorylation (Schaphost et al., 1997). Thrombin induced an increase in pY397-FAK by 10 min of stimulation rising over the time-course under investigation i.e. 60 min (Shikata, 2003) and 120 min (Hollinstat, 2005). Similarly in thrombin-stimulated EA.hy926 cells a small increase in pY397-FAK was induced after 10 min and pY397-FAK was highest at the end of the time-course (60 min).

In contrast to pY397-FAK, increases in pY576-FAK were reported to be induced at different times by the two groups. While Hollinstat et al., (2005) reported the induction to occur after 10 min of exposure to thrombin, Shikata et al. (2003) did not observe increases in pY576-FAK until 30 min of stimulation. In agreement with the Hollistat et al. findings were the observations made by van Nieuw Amerogen et al. (2004), who reported the simultaneous induction of pY397-FAK and pY576-FAK after 10 min of
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thrombin stimulation in HUVECs. Similarly, EA.hy926 cells were observed to respond to thrombin with a small increase in pY576-FAK levels after 10 min of stimulation coinciding in terms of timing with increases in pY397-FAK.

Even though changes in thrombin-induced FAK phosphorylation in EA.hy926 cells were not statistically significant, the observed trends correlated quite well with published results, using other endothelial cell types. In addition to the phosphorylation of specific tyrosine residues an increase in the levels of pY925-FAK in response to thrombin was reported (not investigated in EA.hy926 cells), following a similar profile to the phosphorylation of Y397-FAK (Shikata et al., 2003).

4.6.2 Shear stress-induced FAK phosphorylation
Phosphorylation of FAK has also been reported to occur in response to laminar shear stress. An overall increase in tyrosine phosphorylation of the protein in BAECs and HUVECs by 2 min of shear stress application (12 dyn/cm²) (Ishida et al., 1997).

More specifically, an increase in pY397-FAK has been reported to occur in BAECs after 10 min exposure to 12 dyn/cm² (Li et al., 2002) and in PAECs response to 25 dyn/cm² (Zaidel-Bar et al. 2004). In accordance with these data, increases in pY397- and pY576-FAK were observed in response to high shear stress application in BAECs and HUVECs, and in response to low shear stress in EA.hy926 cells. In contrast, no changes in FAK phosphorylation were induced in HUVEC exposed to low shear stress. Similarly, levels of pY397-FAK were reported to remain unchanged in human lung endothelial cells responding to 15 dyn/cm² laminar shear stress (Shikata et al., 2004). Yet, under these conditions, induction of pY576-FAK was occurred. In contrast, an increase in the phosphorylation of FAK at both Y397 and Y576 residues was reported in response to cyclic stretch (Shikata et al., 2004), implying that differential FAK phosphorylation on specific sites occurs, depending on the type of stress applied. In addition to stimulus specificity though, endothelial cell responses to shear stress may differ in terms of the cell type under investigation or the magnitude of flow applied.
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The effect of low shear stress on endothelial cells has not previously been described. In the current investigation, low shear stress was observed to exert opposite effects on the phosphorylation of FAK in EA.hy926 cells, where an increase in pY397-FAK and pY576-FAK was observed, compared to HUVECs in which, as mentioned before, no change was induced in the phosphorylation of either FAK residue.

4.6.3 The connection between pY397-FAK and pY576-FAK

As discussed in chapter 4.2, the phosphorylated status of the two FAK residues appeared to depend on one another irrespective of cell type or type of stimulation, since in response to stimulation the phosphorylation of both or neither of the residues was induced. This is consistent with published data with the exception of human lung endothelial cells (Shikata et al., 2004).

Comparisons between timings of pY397-FAK and pY576-FAK induction in response to stimulation revealed that pY397-FAK and pY576-FAK either increased simultaneously or that pY576-FAK induction followed that of pY397-FAK. It is nevertheless possible that phosphorylation of Y397-FAK always preceded that of Y576-FAK. If the delay in response was less than a min, then it would not have been detectable under the conditions investigated. Looking at earlier and more frequent time-points could in the future help distinguish between small delays and simultaneous stimulation.

These observations can be well explained by the model put forward by Short et al., (1998) who reported that upon stimulation, either by β1/β3 integrins or by regulatory peptides and lipids acting on G-protein coupled receptors, FAK gets autophosphorylated at the Y397 residue, thereby exposing an SH2-binding domain for the binding of the cytoplasmic kinase Src. It has been proposed that upon binding to FAK, Src is responsible for the phosphorylating of tyrosine residues on proteins in its proximity including FAK (Comillon, 2003). Although it has not been proven it is possible that Y576-FAK is a substrate of Src kinase, and thus the increase of pY397-FAK should precede that of pY576-FAK.
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4.6.4 Thrombin- and shear stress- induced Src activation
Phosphorylation of Src at Y416 is indicative of Src activation as a kinase. Transient phosphorylation of Src has been previously reported in HUVECs exposed to 5 U/ml of thrombin (Biji, 2007), after 5 min and down-regulated by 120 min of stimulation. Similarly, pY416-Src levels were observed to increase in EA.hy926 cells as a result of thrombin (2 U/ml) stimulation, but down-regulation did not occur within the 60 min time-course investigated.

In contrast, transient activation of Src occurred in response to shear stress as indicated by the BAEC and EA.hy926 cell responses to high and low shear stress application respectively. Rapid shear stress-induced increases in pY416-Src levels (2 min) were observed in both BAECs as well as EA.hy926 cells as a result of shear stress application. Probably due to differences in shear stress magnitude, cell type or the combination of the two, pY416-Src induced changes were much more abrupt in BAECs than in EA.hy926 cells where after the induced peak levels of the phosphorylated protein declined gradually with time. Interestingly, Src kinase activity has been reported to increase within seconds (0.5 min) in endothelial cells exposed to shear stress (Okuda et al., 1999 check to see details of flow).

4.6.5 The connection between pY416-Src and pY576-FAK
Comparisons between the timing of the pY416-Src and pY576-FAK indicate that phosphorylation of Y576-FAK could be the result of the action of Src kinase, yet do not prove it. Initial increases in pY416-Src (indicative of Src activation) either preceded or coincided with increases in pY576-FAK levels. It would have been interesting to determine the effect of shear stress (low and high) on pY416-Src in HUVECs especially under low shear stress, where levels of both pY397- and pY576-FAK decreased in response to stimulation.

4.6.6 Thrombin and shear stress effects on paxillin phosphorylation
Both thrombin and shear stress have been reported to induce the phosphorylation of Y31-paxillin and Y118-paxillin. Increases in the phosphorylation of both residues in HUVECs
have been reported to occur within 1 min and maintained until 30 min in response to 12 dyn/cm² shear stress (Mattiussi et al., 2006). Early induction (1 min) of pY31- and pY118-paxillin was also reported in PAECs response to thrombin (Hollinstat et al., 2005). In contrast, in the current investigation, both thrombin and shear stress induced an increase in pY118-paxillin but a reduction in pY31-paxillin levels. These changes occurred early in response to shear stress (2 min) but later in response to thrombin (10 min).

4.6.7 The connection between FAK, Src and Paxillin
In the current investigation a strong correlation was observed between the phosphorylation status of FAK and paxillin, more specifically between pY397-FAK and pY118-paxillin. These two phosphorylation sites followed almost identical timings of phosphorylation changes in response to low shear stress in EA.hy926 cells, in BAECs and HUVECs exposed to high shear stress as well as in the thrombin response in EA.hy926 cells. Interestingly, in all conditions, where pY397-FAK increased and was maintained throughout the time-course, levels of pY118-paxillin also increased and remained higher than those of the control. The only exception was in HUVECs exposed to low shear stress where pY397-FAK did not change.

This agrees with a model where, following FAK autophosphorylation and the binding of Src, the FAK-Src complex is able to recruit and phosphorylate paxillin (Burridge et Al., 1992; Hanks et al., 1992; Plopper and Ingber, 1993). Although in vitro FAK can phosphorylate Y118-paxillin (Plopper and Ingber, 1993) Src has also been implicated in this phosphorylation (Ishibe et al., 2003). The specific staining of pY118-paxillin at focal adhesions in unstimulated as well as stimulated EA.hy926 cells and HUVECs, implies that paxillin is locally phosphorylated at sites of adhesion.

A physical association between paxillin and FAK has been reported in PAECs after thrombin stimulation (Shikata et al., 2003). The FAK binding site of paxillin encompasses the Y31 residue, but the association of the two proteins is not dependent on the phosphorylation of Y31-paxillin (Mattiussi et al., 2006). Consequently, recruitment of
paxillin to the FAK-Src complex and phosphorylation of Y118-paxillin should not be inhibited by the lack of phosphorylation or even dephosphorylation of Y31-paxillin as observed here in response to stimulation.

Nevertheless, the dephosphorylation of Y31-paxillin that was observed under all conditions investigated irrespective of the cell type or type of stimulation is contradictory to published observations on the effect of thrombin or shear stress on the phosphorylation of this residue; as described above both thrombin and shear stress have been reported to induce increases in pY31-paxillin levels with an identical phosphorylation profile to pY118-paxillin (Hollinstat et al., 2005; Mattiussi et al., 2006). This discrepancy could be due to the use of different cell types and/or different experimental conditions. There is no evidence that phosphatases are activated by mechanical forces (Berk et al., 1999), but it is likely that both tyrosine as well as serine/threonine phosphatases would be activated. Zaidel-Bar et al. (2004) have shown localized dephosphorylation and phosphorylation of Y118-paxillin at upstream and downstream (with respect to flow direction) ends in PAECs, illustrating that phosphatases could be activated in response to shear stress and in addition that their function could be specific in terms of localization. The overall decrease in pY31-paxillin observed here could therefore be the result of the action of a phosphatase activated in response to stimulation and recruited to the newly formed complex between paxillin, Src and FAK or even due to the action of activated phosphatases at specific locations within the cell.

4.6.8 Phosphorylation profiles and connection to previous observations

Other than a small decrease in stress fibre content, neither HUVECs responding to high shear stress nor EA.hy926 cells responding to low shear stress showed changes in the cytoskeleton (chapter 3). However, they clearly responded biochemically and in similar ways. Distinct characteristics of the effect of shear stress shared between these two conditions include increased phosphorylation of Y397-FAK, Y576-FAK, Y416-Src (chapter 4) and the lack of MLC phosphorylation and stress fibre formation and no change in tubulin acetylation (chapter 3). As similar trends in the phosphorylation of FAK residues and Src activity occurred in BAECs exposed to high shear stress, it would...
be interesting in the future to compare their response in morphology, MLC phosphorylation or tubulin acetylation. Nevertheless, the data provided on BAECs indicates that similar phosphorylation responses to shear stress are shared between veins and arteries. In contrast, HUVEC exposed to low shear stress (1.6 dyn/cm²) did not respond with changes to Y397-FAK or Y576-FAK phosphorylation (chapter 4) but did show increased MLC phosphorylation levels, stress fibre formation and tubulin deacetylation (chapter 3). These results indicate that FAK phosphorylation is probably not linked to cytoskeletal changes for the conditions investigated or even that the lack of FAK phosphorylation correlates with cytoskeletal changes.

Morphologically, thrombin-induced changes in EA.hy926 cells were most similar to the response of HUVEC to 1.6 dyn/cm² (chapter 3), yet biochemically the response resembled more that of EA.hy926, HUVEC and BAECs exposed to low, high and high shear stress respectively. Interestingly, despite all the differences between the responses, the thrombin and shear stress effect in increasing pY118-paxillin and decreasing pY31-paxillin was consistent in all conditions irrespective of cell type or shear stress magnitude, suggesting that paxillin phosphorylation is not caused by or does not mediate cytoskeletal changes.

In summary, the data obtained indicate that changes in the phosphorylation of focal adhesion proteins do not correlate with cytoskeletal responses to either chemical or mechanical stimuli. Based on the observed timings of focal adhesion protein phosphorylation induced in response to stimulation, it could be hypothesized that integrin activation and downstream signaling is a direct effect of shear stress application but indirect for thrombin.
5 The role of calpain in EA.hy926 cell responses

5.1 Introduction
Calpain-1 and calpain-2 are two members of the calpain family, cytoplasmic cysteine proteases, whose activity is dependent upon the availability of μM and mM calcium concentrations respectively (Goll et al., 2002). Reported substrates of calpains include several focal adhesion proteins such as FAK (Cooray et al., 1996), Src (Oda et al., 1993) and talin (Beckerle et al., 1987). In addition to proteolysis, calpain has been linked to changes in protein tyrosine phosphorylation (Robles et al., 2003). Through their proteolytic function calpains have been implicated in the regulation focal adhesion complex disassembly downstream of microtubules (Bhatt et al., 2002) thereby playing an important role in cell processes such as cell migration in various cell types (see Chapter 1.10.3) and regulation of trans-endothelial cell migration under shear stress conditions (Hussain et al., 2005).

Thrombin and shear stress have been reported to induce calcium influx in endothelial cells (Vanhauwe et al., 2002; Resnick et al., 2003; Tseng et al., 1995). The effect of calpain inhibition was therefore investigated in EA.hy926 cells. The role of calpain in various signalling pathways has been broadly investigated using the cell permeable inhibitors, calpeptin or calpain inhibitor-I (ALLN), which in addition to calpain have also been reported to inhibit other proteases such as the cathepsin family (Donkor, 2000). In the current investigation, the effect of both inhibitors on calpain activity and on the thrombin-induced morphological and biochemical changes reported in previous chapters were investigated.

In an attempt to overcome the limitation of unspecific inhibition of other proteases by the inhibitors, siRNA techniques were employed which enabled investigations regarding the specific role of held by the two calpain isoforms, calpain-1 and calpain-2, in signalling pathways.
5.2 Calpain expression in EA.hy926 cells

As the function of calpain has not to date been investigated in EA.hy926 cells, calpain expression in the specific cell-line had to be determined (Figure 5.1). EA.hy926 cells expressed calpain-1 and calpain-2 isoforms.

![Calpain expression in EA.hy926 cells](image)

**Figure 5.1: Calpain expression in EA.hy926 cells**

Confluent EA.hy926 cells were lysed in sample buffer, electrophorressed on a 4-12% polyacrylamide gel and then probed with antibodies to calpain-1 or calpain-2. Three independent samples of untreated western blotted EA.hy926 cells (lanes 1-3) were tested with each antibody.

5.3 The effect of thrombin and calpain inhibitors on calpain activity

Although calcium influx has been reported in endothelial cells in response to thrombin (Vanhauwe et al., 2002), the effect of the specific thrombin concentration (2 U/ml) on the EA.hy926 cells has not been investigated. A calcium assay kit was therefore purchased (Invitrogen) in order to observe the effect of thrombin on calcium influx in EA.hy926 cells. Calcium ionophore (A23187) was used at 10 μM concentration (Cao et al., 2001) to provide a positive control by inducing maximum calcium influx, while the calcium chelator EGTA used at 1.5 mM concentration to provide the negative control. Unfortunately, great variability was observed between experiments including positive and negative controls, and thus it was not possible to determine whether thrombin induced calcium influx in EA.hy926 cells.

As the main interest of the investigation was the induction of calpain activity in response to stimulation, thrombin-induced calcium influx in EA.hy926 cells was not further investigated and calpain activity assays were instead employed to investigate the effect of stimulation on calpain activity.
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Figure 5.2: The effect of thrombin and calpain inhibitors on calpain activity

After specified cell treatments, calpain activity of cells was assayed and expressed relative to calpain activity of untreated cells. EA.hy926 cells were incubated with 1.5 mM EGTA for 30 min as a negative control. Active calpain-1 suspended in extraction buffer was used as a positive control (see section 2.2.4). 

a) EA.hy926 cells were stimulated with thrombin (2 U/ml) or calcium ionophore A23187 (10 mM) for the indicated time-periods. 
b) EA.hy926 cells were incubated with different concentrations of calpeptin or calpain inhibitor-1 (ALLN) or both for 30 min prior to the assay. 
c) EA.hy926 cells were stimulated with thrombin in the presence and in the absence of both calpain inhibitors (CIs), 17 mg/ml each. Cells were incubated with the CIs for 30 min prior to stimulation. 

The graphs represent the average of three experiments +/- s.e.m. Statistical significance was determined using ANOVA and Student’s t-test (* and * p<0.05). * denotes statistically significant differences of treated EA.hy926 cells relative to the 0 time-point of the particular treatment and * denotes statistically significant differences between different treatments at individual time-points.
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5.3.1 Thrombin-induced transient activation of calpain

In order to investigating the role of calpain in endothelial response to thrombin, its effect on calpain activity was investigated. Confluent EA.hy926 cells were stimulated with thrombin and calpain activity was determined. The assay measured the fluorescence emitted by AFC, the cleaved product of the calpain substrate (Ac-LLY-AFC) produced upon incubation of the substrate with cell lysates containing active calpain (see Chapter 2.2.4). Calcium ionophore (A23187) and EGTA were used as controls as they have been reported to induce and inhibit the activity of calpain respectively (Zhu et al., 2007; Wu et al., 2007).

EA.hy926 cells responded to both thrombin and calcium ionophore by a transient activation of calpain (Figure 5.2a). The maximal increase in calpain activity was 35% for thrombin and 75% for calcium ionophore and occurred within seconds of stimulation. Although the initial increase in calpain activity occurred after 10 seconds of stimulation with both stimuli, in the case of thrombin a statistically significant increase was not achieved until after 20 seconds of stimulation as opposed to 10 seconds for calcium ionophore. The increase in calpain activity after 10 seconds of stimulation was significantly higher for calcium ionophore compared to thrombin. Treatment of cells with EGTA, used to chelate calcium, reduced calpain activity, whereas addition of active calpain-1 to the extraction buffer induced calpain activity (Figure 5.2a).

Calpain activity is therefore regulated by calcium levels in endothelial cells and within seconds of thrombin stimulation the activity of calpain increased by a significant amount, indicating that calpain could have a role in endothelial cell responses to thrombin.

5.3.2 Calpain inhibition by pre-treatment with calpain inhibitors

In order to determine the optimal concentration of calpain inhibitors for the maximum effect on calpain activity, calpain activity assays were carried out in EA.hy926 cells in the presence of different concentrations of individual calpain inhibitors (Figure 5.2b) as well as together (Figure 5.2c).
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When used independently, each calpain inhibitor led to a 30 to 40% reduction in calpain activity (Figure 5.2b). Pre-treatment of EA.hy926 cells with both inhibitors, each at 17 µg/ml.

Longer pre-treatment of EA.hy926 cells with the inhibitors (1 hour) lead to significant amounts of cell detachment. Thus, it was decided that in order to investigate the role of calpain, EA.hy926 cells would be incubated with both calpain inhibitors (17 µg/ml each) for 30 min prior to the beginning of an experiment. Inhibitors were present throughout experiments, after to the point of lysis of fixation.

5.3.3 Calpain inhibitors significantly reduced thrombin-induced calpain activity

Since even after treatment of cells with inhibitors active calpain was present (approximately 50%), calpain activity was determined in thrombin-stimulated EA.hy926 cells pre-treated with calpain inhibitors, in order to determine whether the inhibitors reduced thrombin-stimulated calpain activity (Figure 5.2c).

The thrombin-induced statistically significant transient activation of calpain occurred even in the presence of calpain inhibitors, yet calpain activity levels were lower than control cells at all times. The peak in calpain activity exhibited by thrombin-stimulated EA.hy926 cells was reduced by only 20% reduction by treatment with inhibitors. Comparisons of activity, between calpain inhibitor-treated and untreated cells at individual time-points revealed a reduction in calpain activity as a result of calpain inhibition. It is also possible that the activity of other proteases able to recognize and cleave the synthetic substrate is measured by the assay.

The reduction in calpain activity by calpain inhibitors could affect the thrombin response in endothelial cells.
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5.4 Morphological Changes in Response to Calcium Ionophore
Since, calpain activation is a calcium-dependent process, the effect of calcium ionophore in EA.hy926 cells and HUVECs was investigated, in order to deduce whether the morphological changes observed in thrombin-stimulated cells could be the result of an increase in intracellular calcium levels.

In response to stimulation with the calcium ionophore A23187, endothelial cell contraction was observed leading to intracellular gap formation by 30 min of stimulation (Figure 5.3). VE-cadherin staining was present at sites of cell attachment both prior and after stimulation. As was the case with thrombin (see Chapter 3.9), after stimulation with A23187 some loss of peripheral VE-cadherin staining was observed without a particular increase in intracellular levels. For F-actin and pY118-paxillin, the response of HUVECs (Figure 5.3a) to calcium ionophore was more similar to the thrombin response than EA.hy926 cells (Figure 5.3b). An increase in cortical F-actin and the generation of elongated thicker focal adhesions was observed in A23187-treated HUVECs, but not in EA.hy926 cells. However, elongation of focal adhesions was not clearly observed in thrombin-stimulated EA.hy926 cells either (see Chapter 4.5.2). The morphological changes induced by calcium ionophore stimulation in endothelial cells has some similarities to thrombin-stimulated cells suggesting that thrombin-induced morphological changes may be in part the result of an increase in cytosolic calcium levels.

5.5 The effect of calpain inhibition on thrombin- or A23187- induced morphology
In order to investigate the effect of calpain inhibition on the thrombin pathway, EA.hy926 cells were stimulated with thrombin in the presence as well as in the absence of calpain inhibitors. The effect of calpain inhibition on the thrombin-induced junctional and cytoskeletal (actin and microtubule) reorganization (described in Chapter 3) was investigated. Calpain inhibitor effects on calcium ionophore-induced morphology were also investigated in order to observe the extent to which inhibition of calpain affects calcium signalling.
Figure 5.3: The effect of calcium ionophore stimulation on endothelial cells

Confluent monolayers of a) HUVECs and b) EA.hy926 cells were stimulated with the calcium ionophore A23187 (10 mM) for the indicated time-periods and stained for F-actin (green), VE-Cadherin (blue) and pY118-paxillin (red). Results are representative of at least three experiments. Bar= 20 μm. Gray arrows point to focal contacts.
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5.5.1 Calpain inhibition induces endothelial cell contraction

EA.hy926 cells stimulated with thrombin in the absence and presence of calpain inhibitors were visualized using phase-contrast microscopy (Figure 5.4). In the presence of calpain inhibitors, EA.hy926 cells were observed to contract. Although thrombin induced contraction of the endothelial cell monolayer was not evident in the absence of the inhibitors, in their presence it was. Whether thrombin-induced contraction of EA.hy926 cells in the absence of inhibitors was not detected due to the low magnification or the use of plastic instead of glass (used in immunofluorescence experiments; Figure 5.6) has not been determined.

The F-actin staining of unstimulated EA.hy926 cells in the presence and absence of calpain inhibitors (Figure 5.6) revealed that calpain inhibitor-induced endothelial cell rounding did not involve the same reorganization of actin filaments as thrombin. EA.hy926 cells responded to calpain inhibitors by contracting without increasing their content of cortical F-actin. The organization of actin filaments in calpain inhibitor treated endothelial cells resembled more the calcium ionophore- induced contraction though not identical to it. Even though the enhancement of thrombin-induced cell contraction observed under phase-contrast microscopy (Figure 5.4) in the presence of calpain inhibitors was not observed in the immunofluorescence experiments, the effect was very clear in calcium-stimulated EA.hy926 cells (Figure 5.6b).
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Figure 5.4: Calpain inhibitors induced cell rounding further enhancing the effect of thrombin. Ea.hy926 cells were treated with 2 U/ml of thrombin in the presence or absence of CIs (calpeptin and calpain inhibitor-I, 17 μg/ml each) for 60 min and were observed by phase-contrast time-lapse microscopy (section 2.2.3.2). Shown are frames of the 0 min and 60 min time-points. Representative of three experiments. Red arrows indicate gaps in the endothelium.

Figure 5.5: The effect of calpain inhibitors on MLC phosphorylation. Confluent EA.hy926 cells were stimulated with thrombin (2 U/ml) in the presence or absence of calpain inhibitors (CIs), calpeptin and calpain inhibitor-I, 17 μg/ml each. a) lysates were electrophoresed on the same gel and probed with antibodies to pT18S19-MLC. ERK was used as a loading control. Representative of three experiments. b) pMLC levels of treated cells relative to pMLC levels of untreated control cells, mean of three independent experiments +/- s.e.m. Statistical significance was determined using ANOVA and Student’s t-test (* p<0.05). * denotes statistically significant differences of calpain inhibitor-treated cells relative to cells not treated with CIs for individual time-points.
Figure 5.6: The effect of calpain inhibition on the actin cytoskeleton

EA.hy926 cells were stimulated with a) 2 U/ml of thrombin or b) 10 mM of calcium ionophore (A23187) for the time-periods indicated, in the presence or the absence of calpain inhibitors (CIs). Cells were fixed and stained for F-actin. Cells were incubated with calpeptin and calpain inhibitor-I (17 µg/ml each) for 30 min prior to stimulation. Bar= 20 µm.
Because calpain inhibitors induced cell rounding their effect on MLC phosphorylation, reported to be involved in the regulation of cell contraction, was investigated (Figure 5.5). Increased MLC phosphorylation was detected using a phospho-specific antibody recognizing phosphorylated Ser19 and Thr18. A small increase in MLC phosphorylation was observed in response to thrombin stimulation both in the presence and absence of calpain inhibitors (also see Chapter 3.3.1). While there was a consistent increase in all three independent experiments differences in the minor changes were not significant. The pMLC levels in inhibitor-treated EA.hy926 cells were slightly higher than in cells without calpain inhibitors. After 60 min of thrombin stimulation the difference in pMLC levels of inhibitor-treated and non-treated cells was approximately 40%, a difference that proved statistically significant. Although calpain inhibitors had no major effect on MLC phosphorylation, it is possible that the significance of the minor increases observed would only become evident at later time-points of stimulation, beyond 60 min.

5.5.2 Calpain inhibition induces re-organization of VE-Cadherin

EA.hy926 cells were stimulated with thrombin or calcium ionophore in the presence or in the absence of calpain inhibitors and stained for VE-Cadherin (Figure 5.7). VE-cadherin was localized at areas of cell-cell contact and this was not altered by calpain inhibitors. However, its localization was discontinuous and less linear in areas where cells were probably contracting and pulling apart from other cells. This was higher in calpain inhibitor-treated cells particularly in the presence of A23187 or thrombin.
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Figure 5.7: The effect of calpain inhibition on VE-cadherin localization

EA.hy926 cells were stimulated with a) 2 U/ml of thrombin or b) 10 mM of calcium ionophore (A23187) for the time-periods indicated, in the presence or the absence of calpain inhibitors (CIs). Cells were fixed and stained for VE-cadherin. Cells were incubated with calpeptin and calpain inhibitor-I (17 μg/ml each) for 30 min prior to stimulation. Bar= 20 μm.
5.5.3 Calpain inhibition induces reorganization of the microtubule network

EA.hy926 cells were stimulated with thrombin or calcium ionophore in the presence or absence of calpain inhibitors and stained for β-tubulin (Figure 5.8). Under unstimulated conditions reorganization and blebbing at the cell periphery occurred in the presence of calpain inhibitors. Occasionally blebbing was observed in thrombin stimulated EA.hy926 cells in the absence of calpain inhibitors. A decrease in the number and possibly length of microtubule filaments was detected after 5 min of thrombin and A23187-treated EA.hy926 cells in the presence of calpain inhibitors which seemed to recover by 30 min of stimulation. However, no quantifications were carried out, hence the observation could not be confirmed.

As described in Chapter 3, thrombin reduced acetylated tubulin levels in EA.hy926 cells. In order to determine whether the reorganization of the microtubule network observed correlated with a change in the acetylation of tubulin, the effect of calpain inhibitors on thrombin-induced changes of acetylated tubulin levels was investigated (Figure 5.9). Under unstimulated conditions, calpain inhibitor-treated cells exhibited approximately 20% lower levels of acetylated tubulin than control cells. Yet, calpain inhibitors prevented the thrombin-induced decrease in acetylated tubulin between 5 and 30 min. It is possible that the contraction of endothelial cells could be responsible for the lower levels of acetylated tubulin observed as a result of calpain inhibitor treatment in unstimulated endothelial cells and that calpain is required for thrombin-induced deacetylation of tubulin.
The role of calpain in EA.hy926 cells

Figure 5.8: The effect of calpain inhibition on the microtubule cytoskeleton

EA.hy926 cells were stimulated with a) 2 U/ml of thrombin or b) 10 mM of calcium ionophore (A23187) for the time-periods indicated, in the presence or in the absence of calpain inhibitors (CIs). Cells were fixed and stained with β-tubulin. Cells were incubated with calpeptin and calpain inhibitor-I (17 µg/ml each) for 30 min prior to stimulation. Bar= 20 µm. Red arrows point to “blebbing”. Representative of at least three experiments.
The role of calpain in EA.hy926 cells

Figure 5.9: The effect of calpain inhibitors on tubulin acetylation
Confluent EA.hy926 cells were stimulated with thrombin (2 U/ml) for the time-periods indicated in the presence or absence of calpain inhibitors (CIs: calpeptin and calpain inhibitor-1, 17 µg/ml each, for 30 min prior to stimulation. a) Lysates were electrophoresed on the same 4-13% SDS-PAGE gel and probed with antibodies to acetylated tubulin (AcTub). ERK was used as a loading control. Representative of three experiments. b) AcTub levels of treated cells relative to levels of untreated control cells the mean of three experiments +/- s.e.m. The significance of the differences in AcTub were determined using ANOVA and Student’s t-test (* and * P<0.05). * denotes statistically significant differences of stimulated cells relative to the zero time-point for each condition (with and without CIs). * denotes statistically significant differences of calpain inhibitor-treated cells relative to cells not treated with CIs for individual time-points.
5.6 The effect of calpain inhibition on focal adhesion proteins

In platelets, calpain has been linked to alterations in the balance between the activities of tyrosine kinases and phosphatases, thereby regulating the phosphorylated state of different proteins including focal adhesion proteins (Pain et al., 1999; Robles et al., 2003). The effect of calpain inhibitors on thrombin-induced changes in the phosphorylation of FAK, Src and paxillin was therefore investigated in EA.hy926 cells to determine whether a similar role is played by endothelial calpains.

5.6.1 Calpain inhibitors and thrombin-induced effects on focal adhesion proteins

Under unstimulated conditions in the presence of calpain inhibitors, levels of pY397-FAK were slightly higher than in the absence of calpain inhibitors, while those of pY576-FAK were slightly lower (figure 5.10). The changes were small and statistically non-significant but they were consistent between experiments. However, calpain inhibition was observed to prevent thrombin-induced phosphorylation of both FAK residues.

A reduction in pY416-Src levels occurred in the presence of calpain inhibitors (Figure 5.11). Under unstimulated conditions, calpain inhibitor-treated cells contained approximately 30% less phosphorylated protein. Calpain inhibitors also prevented the thrombin-induced increase in pY416-Src (also see Chapter 4.3.1).

As discussed in Chapter 4 a correlation between stimuli-induced increases in pY576-FAK levels and pY416-Src levels was observed, suggesting that phosphorylation of the specific FAK residue was the result of the action of phosphorylated Src. Thus, the inability of thrombin to induce the phosphorylation of Y416-Src in the presence of calpain inhibitors could explain the lack of thrombin-induced increases in pY576-FAK.

Comparisons between the timing of thrombin-induced changes of FAK, Src and paxillin phosphorylation (also see Chapter 4) indicated that thrombin-induced paxillin phosphorylation could be dependent upon the phosphorylation of FAK and Src (also Chapter 4.4.2). Like pY397-FAK, levels of pY118-paxillin were slightly higher in the presence of calpain inhibitors between 0 and 10 min of thrombin stimulation and lower at
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later time-points (Figure 5.12a). The differences were not statistically significant, but consistent between three different independent experiments. Calpain inhibitors also prevented the thrombin-induced increase and decrease in pY118-paxillin and pY31-paxillin levels respectively (also see Chapter 4.4.2). In the presence of calpain inhibitors, pY118-paxillin levels remained constant, whereas pY31-paxillin slightly increased, in response to thrombin.

These results suggest that calpain is required for the thrombin-induced changes in the phosphorylation of FAK and paxillin and is able to regulate the phosphorylation of Y416-Src.

5.6.2 The effect of calpain inhibitors on focal adhesions

As mentioned before, in addition to affecting the phosphorylation of focal adhesion proteins, calpains have been implicated in the regulation of focal adhesion disassembly (Bhatt et al., 2002). Inhibition of calpain has been reported to lead to the formation of strong focal adhesions at cell periphery (Dourdin et al., 2001; Bhatt et al., 2002).

The effect of calpain inhibitors on the localization of pY118-paxillin was therefore investigated in thrombin-stimulated EA.hy926 cells (Figure 5.12b). Calpain inhibition did not affect the localization of pY118-paxillin to focal adhesions. In the presence of the inhibitors, fewer and larger (thicker) focal adhesions were observed at both 5 min and under unstimulated conditions, located mainly at the cell periphery. No obvious alterations were observed on the numbers or size of focal adhesions in response to thrombin in the presence of calpain inhibitors, while larger more elongated focal adhesions were observed in the absence of the inhibitors in HUVECs (see Chapter 4.5.2).

The 50% reduction in calpain activity was thus sufficient to generate the formation of larger yet fewer focal adhesions in EA.hy926 cells, to slightly increase the levels of pY397-FAK, pY118-paxillin and pY31-paxillin under unstimulated conditions, yet significantly reduce the levels of pY416-Src and slightly reduce pY576-FAK and to abolish thrombin-induced alterations in the phosphorylation status of FAK, paxillin and
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Src. In EA.hy926 cells, therefore, calpain appears to play a role in the thrombin signalling pathway possibly by regulating focal adhesion disassembly.

5.7 The effect of calpain inhibition on Rho protein activity

In previous experiments, thrombin was observed to stimulate the activity of RhoA while it had no effects on the activity of Rac1 (see Chapter 3.4). Based on the conclusion that calpain plays a role in the thrombin signalling pathway, the effect of calpain inhibitors on RhoA and Rac1 activity was investigated. Unfortunately, RhoA pull-down experiments carried out in the course of the investigation were unsuccessful and no conclusions could be made regarding the effect of calpain inhibition on RhoA activity in EA.hy926 cells.

Nevertheless, from the Rac1 pull-down experiments (Figure 5.13) it was observed that calpain inhibition led to an increase in Rac1 activity, yet in response to thrombin, Rac1 activity remained unaltered.
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Figure 5.10: The effect of calpain inhibitors on FAK phosphorylation
Confluent EA.hy926 cells were stimulated with thrombin (2 U/ml) for the time-periods indicated in the presence and in the absence of calpain inhibitors (CIs). Cells were incubated with the inhibitors (calpeptin and calpain inhibitor-1, 17 µg/ml each) for 30 min prior to stimulation. a) lysates were electrophoresed on the same gel and probed with antibodies to pY397-FAK or pY576-FAK. ERK was used as a loading control for the quantifications. Representative of three experiments. b) pFAK levels of treated cells relative to levels of untreated control cells, the mean of three experiments +/- s.e.m. The significance of the differences in pFAK levels in stimulated cells relative to the zero time-point for each condition (with and without CIs) as well as the significance of the differences between the two conditions for each time-point were analyzed using ANOVA and Student’s t-test. None of the changes proved statistically significant.
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Figure 5.11: The effect of calpain inhibitors on Src phosphorylation
Confluent EA.hy926 cells were stimulated with thrombin (2 U/ml) for the time-periods indicated in the presence and in the absence of calpain inhibitors (CIs). Cells were incubated with the inhibitors (calpeptin and calpain inhibitor-1, 17 μg/ml each) for 30 min prior to stimulation a) lysates were electrophoresed on the same 4-12% gel and probed with antibodies to pY416-Src. ERK was used as a loading control for the quantifications. Representative of three experiments. b) shows pSrc levels of treated cells relative to pSrc levels of untreated control cells the average of three experiments +/- s.e.m. Statistical significance was determined using ANOVA and Student’s t-test (*and ** P<0.05). * denotes statistically significant differences of stimulated cells relative to the zero time-point for each condition (with and without CIs) ** denotes statistically significant differences of calpain inhibitor-treated cells relative to cells not treated with CIs for individual time-points.
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Confluent EA.hy926 cells were stimulated with thrombin (2 U/ml) in the presence and in the absence of calpain inhibitors (CIs, 17 μg/ml each). a) After treatment cell were lysed, electrophoresed on the same 4-12% SDS-PA gel and probed with antibodies to pY118-paxillin or pY31-paxillin. ERK was used as a loading control. The graph shows pPaxillin levels of treated cells relative to levels of untreated control cells, the mean of three experiments +/- s.e.m. Statistical significance was determined using ANOVA and Student’s t-test (* P<0.05). * denotes statistically significant differences of calpain inhibitor-treated cells relative to cells not treated with CIs for individual time-points. b) After treatment cells were fixed and stained with pY118-paxillin. Representative of at least three experiments. Bar= 20 μm. Arrows point to focal contacts.

Figure 5.12: The effect of calpain inhibitors on paxillin phosphorylation
Figure 5.13: The effect of calpain inhibitors on Rac1 activity

a) Confluent EA.hy926 cells were treated with calpain inhibitors (calpeptin and calpain inhibitor-I, 17 μg/ml each). After treatment Rac-GTP pull-down assays were performed (section 2.2.2.4). After treatment GST-PBD bound proteins were electrophorressed on a 14% SDS-PA gel and probed with antibodies to Rac1; indicating levels of active Rac1. Whole cell lysates were also electrophorressed on a 14% SDS-PA gel and probed with Rac1 indicating levels of total Rac1. b) Confluent EA.hy926 cells were stimulated with thrombin (2 U/ml) for the time-periods indicated in the presence of calpain inhibitors (CIs: calpeptin and calpain inhibitor-I, 17 μg/ml each). After treatment Rac-GTP pull-down assays were performed as described in (section). The GST-PBD bound proteins were electrophorressed on a 14% SDS-PA gel and probed with antibodies for Rac1, indicating levels of active Rac1. Whole cell lysates were also electrophorressed on a 14% SDS-PA gel and probed with Rac1 indicating levels of total Rac1. Graphs next to each blot represent levels of Rac1-GTP relative to untreated controls; the mean of three experiments +/- s.e.m. Statistical significance was determined using ANOVA and Student’s t-test. (p<0.05)
5.8 The differential effects of the two calpain inhibitors

As the calpain inhibitors used in the experiments above target other proteins as well as calpain (Donkor, 2005), the effect of the individual inhibitors on endothelial cells was investigated. Each inhibitor was used at 17 μg/ml, which only achieved approximately 30% inhibition of calpain activity as opposed to 50% when both inhibitors were used (see Figure 5.2).

5.8.1 The effects of calpain inhibitors on the cytoskeleton and junctions

The endothelial cell rounding induced when EA.hy926 cells were treated with both calpain inhibitors was also observed in cells treated with individual inhibitors, but was stronger in the case of calpeptin incubation (Figure 5.14).

VE-cadherin localization at cell-cell junctions persists in EA.hy926 cells whether treated with individual or the combination of calpain inhibitors. Whereas a certain loss of peripheral VE-cadherin staining was detected in cells pulling apart, some cells retained their peripheral VE-cadherin localization irrespective of having lost their cell-cell contact. The increased contraction of calpeptin-treated cells was characterized by a more intense F-actin staining at the cell periphery compared to cells treated with both inhibitors, which was in turn more intense than when calpain inhibitor-I was used on its own.

Peripheral blebbing was clearly observed in cells stained with antibodies to β-tubulin and correlated with cell rounding. Some of these blebs were also visibly, but less clearly, by F-actin staining. There appeared to be fewer microtubules in calpeptin-treated cells or cells treated with both calpain inhibitors, compared to control, or calpain inhibitor-I – treated cells.

The two calpain inhibitors affected the actin and microtubule cytoskeletons in different ways and the observed changes in the morphology of EA.hy926 cells as a result of their incubation with both inhibitors were a mixture of the effects of calpeptin and calpain inhibitor-I.
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Figure 5.14: The effect of calpain inhibitors on the cytoskeleton and junctions
Confluent EA.hy926 cells were incubated with calpeptin or calpain inhibitor-I or the combination of two calpain inhibitors (CIs, 17 μg/ml each) for 30 min after which, cells were fixed and stained for F-actin (left panel) and β-tubulin (middle panel) or VE-cadherin (right panel). Bar= 20 μm.
5.8.2 The effects of calpain inhibitors on MLC phosphorylation, tubulin acetylation

As a consequence of the differential effects of calpain inhibitors on the actin and microtubule cytoskeletons, their effect on MLC phosphorylation (involved in the regulation of cell contraction, Baluk et al., 1997) and on tubulin acetylation (AcTub: indicator of microtubule stabilization) was investigated (Figure 5.15). The two calpain inhibitors affected MLC phosphorylation and tubulin acetylation to quite different extents.

Under unstimulated conditions neither the individual inhibitors nor the combination of inhibitors effected MLC phosphorylation. In response to thrombin, however, statistically levels of pMLC were higher in calpeptin-treated EA.hy926 cells than in control, calpain inhibitor-I or both inhibitors (Figure 5.15a and b). Significant increase in pMLC was induced as early as 2 min after stimulation in calpeptin-treated cells and remained high throughout the time-course. The effect of the presence of both calpain inhibitors on thrombin-stimulated EA.hy926 cells was very similar to that of calpain inhibitor-I, characterized by slight or no increases in pMLC levels in response to thrombin. This was surprising given that blebbing occurred in the presence of both calpain inhibitors (Figure 5.8). EA.hy926 cell treatment with calpain inhibitor-I and both inhibitors, resulted in approximately 30% reduction in acetylated tubulin levels, but results with calpain inhibitor-I were more variable and hence not significant. It is possible that calpain inhibitor-I has no effect on tubulin acetylation and that changes in the presence of both inhibitors are most likely to be the effect of calpeptin, which when used independently significantly decreased acetylated tubulin levels by approximately 40%.
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Figure 5.15: The effect of calpain inhibitors on pMLC and AcTub

Confluent EA.hy926 cells were incubated with calpeptin or calpain inhibitor-I (17 μg/ml each). a) EA.hy926 cells were stimulated with thrombin (2 U/ml) in the presence or absence of inhibitors. Lysates were electrophoresed and probed with antibodies to pMLC. ERK was used as a loading control. Representative of three experiments c) Lysates of EA.hy926 cells treated with the CIs and untreated controls were electrophoresed and probed for acetylated tubulin (AcTub). C: untreated, B: both inhibitors, CI-1: calpain inhibitor-I, CALP: calpeptin. Total tubulin was used as a loading control. b) and d) pMLC and AcTub levels of treated EA.hy926 cells relative to the levels of the untreated controls; the mean of three experiments +/- s.e.m. Statistical singificance was determined using ANOVA and Student’s t-test (*and * P<0.05). * denotes statistically significant differences of stimulated cells relative to the zero time-point for each condition. * denotes statistically significant differences of CI-treated cells relative to cells not treated with CIs for individual time-points.
5.8.3 The effects of calpain inhibitors on focal adhesions

The effect of individual calpain inhibitors on focal adhesions was determined by staining of calpain inhibitor treated EA.hy926 cells with antibodies to pY118-paxillin (Figure 5.16). As discussed earlier, incubation of EA.hy926 cells with both inhibitors (calpeptin and calpain inhibitor-I) led to the generation of fewer and bigger focal adhesions compared to control cells. Calpeptin alone induced a similar but stronger response in EA.hy926 cells, probably indicating the stabilization of focal adhesions. In response to calpain inhibitor-I treatment though, neither an increase in the size nor a decrease in the numbers of focal adhesions was observed.

The scaffolding protein talin, a reported substrate of calpain, also localizes at focal adhesions (Franco and Huttenlocher, 2005). The effect of each calpain inhibitor on talin localization was therefore investigated in EA.hy926 cells (Figure 5.17). Like pY118-paxillin, talin localized to focal adhesions both in the presence and absence of calpain inhibitors, and focal adhesions were larger in the presence of calpeptin but not calpain inhibitor-I.

In the presence of both calpain inhibitors, morphological changes associated with the cytoskeleton and focal adhesions were therefore mostly the consequence of the action of calpeptin rather than calpain inhibitor-I. It would be interesting to determine whether there is a connection between the calpeptin-induced tubulin deacetylation and hence presumably microtubule destabilization and focal adhesion stabilization.
Figure 5.16: The effect of the individual calpain inhibitors on pY118-paxillin

EA.hy926 cells were incubated with calpeptin, calpain inhibitor-I or both, each used at 17 µg/ml for 30 min. Calpain inhibitor-treated and untreated endothelial cells were fixed and stained for pY118-paxillin. Representative of at least three experiments. Bar= 20 µm. The right panels are magnifications of the indicated areas in the left panel. Red arrows point to focal adhesions.
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Figure 5.17: The effect of the individual calpain inhibitors on talin localization
EA.hy926 cells were incubated with calpeptin, calpain inhibitor-I or both, each used at 17 μg/ml for 30 min. Calpain inhibitor-treated and untreated endothelial cells were fixed with methanol and stained with antibodies to talin. Representative of at least three experiments. Bar= 20 μm. The right panel illustrates the magnification of the indicated area in the left panel. Red arrows point to focal adhesions.
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5.9 The effect of RNAi-induced down-regulation of calpain isoform expression

Due to the differences in response to the individual calpain inhibitors, the effect of calpain inhibition on endothelial cells and on the thrombin pathway were investigated by RNAi (see Chapter 2.2.1.3). The aim for this investigation was two fold: a) to deduce whether the two calpains have distinct roles in endothelial cells and/or in the thrombin pathway and b) to study the role of calpains by silencing the expression of both. However, attempts to knock down both calpain isoforms together led to cell death/detachment indicating that calpains are essential to cell survival. Calpain siRNAs were used at 15 nM resulting in efficient (>60%) knock-down of calpain-1 and calpain-2 (Figure 5.18). It was therefore only possible to study the effects of knocking down individual isoforms.

![Calpain-1 and Calpain-2 Western Blots](image)

**Figure 5.18: RNAi induced down-regulation of calpain isoforms**

EA.hy926 cells were transfected with calpain-1 siRNA (CPN1) or calpain-2 siRNA (CPN2). After 48 hours, cells were lysed in sample buffer, electrophorressed on a 4-12% polyacrylamide gel and probed with antibodies to calpain-1 or calpain-2 respectively. Untreated, cells treated with stable fluorescence non-targeting control siRNA (siGLO) and csiRNA (Cs) transfected EA.hy926 cells were used as controls. ERK was used as a loading control.

As mentioned in Chapter 2.2.1.3, all experiments were carried out using siGLO- and csiRNA-transfected cells as controls but the latter was later found to have non-specific side effects in endothelial cells (Dr. Jaime Milan, personal communication). Therefore the effect of calpain-1 and calpain-2 siRNA treatment was only compared to that of siGLO-transfected cells, hence the data for csiRNA is not shown for later Chapters.
5.9.1 Effect of calpains on the cytoskeleton, junctions and focal adhesions

RNAi induced silencing of either calpain-1 or calpain-2 expression, was observed to result in increased numbers of stress fibres (Figure 5.19a). The increase in stress fibre content as a result of siRNA treatment led to the formation larger focal adhesions at the cell periphery, in calpain-2 but not in calpain-1 siRNA-treated EA.hy926 cells (Figure 5.19b). Fewer focal adhesions were observed in the cells treated with calpain-2 or calpain-1 siRNA. Efficient siRNA transfection was only possible in sub-confluent endothelial cells, and thus they had not formed a confluent monolayer at the time of the experiments. It is therefore possible that differences associated with focal adhesion size result from differences in monolayer confluency.

VE-cadherin was localized at sites of cell-cell contact indicating that reduced expression of either calpain-1 or calpain-2 had no effect on the localization of VE-cadherin at cell periphery (Figure 5.20). Due to the predominance of single cells, it was not possible to determine whether thrombin stimulation would result in a loss of junctional integrity.

siRNAs to calpain-1 or calpain-2 did not affect the integrity of the microtubule network (Figure 5.21a). A small decrease in acetylated tubulin levels observed in siRNA treated cells also occurred in siGLO-transfected cells indicating that siRNA (Figure 5.21b). In contrast to calpeptin and similar to calpain inhibitor-I, siRNA transfection did not induced blebbing.

Morphological changes associated with RNAi-induced down-regulation of calpain isoforms were different to those induced by calpain inhibitors, probably indicating non-specific inhibitor action. Both calpain isoforms appeared to regulate the formation of stress fibres. In contrast, the generation of big focal adhesions at the cell periphery observed in inhibitor-treated EA.hy926 cells was probably induced via inhibition of calpain-2 rather than calpain-1.
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Figure 5.19: The effect of calpain isoforms on stress fibres and focal adhesions
EA.hy926 cells were transfected with calpain-1 or calpain-2 siRNA. Green and red transfection indicators (table 2.11) was co-transfected with the siRNA (siGLO). Cells transfected with siGLO alone were used as controls. 48 hours after transfection cells were fixed and stained for a) F-actin b) pY118-paxillin. The lower panel is a magnification of the pY118-paxillin channel of the area indicated on the merge panel. Bar= 20 μm. Red arrows point to focal contacts. Both results are representative of two experiments.
Figure 5.20: The effect of individual calpain isoforms on junctions

EA.hy926 cells were transfected with calpain-1 or calpain-2 siRNA. After 48 hours, cells were stimulated with thrombin (2 U/ml) for 10 min, fixed and stained for VE-cadherin. Representative of two experiments. Red transfection indicator (siGLO) was co-transfected with the siRNA. Cells transfected with siGLO alone were used as controls. Bar= 20 μm.
The role of calpain in EA.hy926 cells

![Image](image_url)

**Figure 5.21: Neither calpain isoform affects microtubules or tubulin acetylation**

a) EA.hy926 cells were transfected with siRNA to calpain-1 or with siRNA calpain-2. Red transfection indicator (siGLO) (table 2.11) was co-transfected with the siRNA. Cells transfected with siGLO alone were used as controls. 48 hours after transfection, cells were stimulated with thrombin (2 U/ml) for 10 min, fixed with PFA and stained with β-tubulin. Representative of two experiments. Bar= 20 μm. b) EA.hy926 cells were transfected with siRNA calpain-1 or with siRNA calpain-2. siGLO transfected and untransfected cells were used as controls. 48 hours after treatment, cells were lysed in sample buffer electrophoresed on a 4-12% SDS-PA gel and probed with antibodies to acetylated tubulin (AcTub). Total tubulin was used as a loading control. The levels of acetylated tubulin in siRNA treated and siGLO transfected cells were quantified and expressed as a ratio of the levels of acetylated tubulin of untransfected cells. The average of two experiments.
5.9.2 Differential effect of the two calpains on thrombin-induced cell contraction

To determine whether calpains affect the morphological response to thrombin, cell spread area and circularity were quantified in EA.hy926 cells before and after thrombin stimulation.

Under unstimulated conditions, calpain-1 siRNA-treated EA.hy926 cells had a similar spread area to siGLO-transfected and untransfected cells, whereas the RNAi-induced down-regulation of calpain-2 significantly increased cell spreading thereby increasing the spread area by approximately 40% (Figure 5.22a). As expected, spread area was reduced upon thrombin stimulation, in both untransfected and mock transfected cells (approximately by 40%). Calpain-1 siRNA-treated cells, exhibited a similar decrease in spread area as the controls, but only a slight reduction (approximately 20%) in cell area was induced in calpain-2 siRNA treated EA.hy926 cells upon thrombin stimulation.

Quantifications of cell circularity (Figure 5.22b) confirmed the association of thrombin-induced cell contraction with cell rounding. Thrombin stimulation significantly increased rounding in both control and calpain-1 siRNA-treated EA.hy926 cells. In contrast, thrombin-induced endothelial cell rounding was not observed in calpain-2 siRNA-treated EA.hy926 cells. In addition to the effects of thrombin, quantifications of cell circularity of unstimulated cells revealed that calpain-1 siRNA-transfected cells had higher circularity, indicating differences in cell shape.

Calpain-2 appeared to have a more important role in the thrombin signalling pathway than calpain-1. The observed inhibition of thrombin-induced cell rounding in response to decreased calpain-2 expression could be the consequence of focal adhesion stabilization. Stabilization would prevent the disassembly of old and formation of new focal adhesions thereby preventing endothelial cells from adopting a new morphology. Under unstimulated conditions, inhibition of both calpain-1 and calpain-2 was observed to regulate cell shape to a certain degree, by inducing cell contraction and spreading respectively.
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Figure 5.22: Effect of the two calpains in thrombin-induced cell contraction

Area and circularity of EA.hy926 cells transfected with calpain-1 (CPN1) or calpain-2 (CPN2) siRNAs before and after 10 min stimulation with thrombin (2 U/ml) were calculated using ImageJ software (see section 2.2.5.2). Four independent experiments were used for the quantifications (n>30 for each experiment) a) spread area of cells before and after thrombin stimulation expressed as a ratio to the spread area of untransfected, non-stimulated EA.hy926 cells. b) absolute circularity values of cells, under unstimulated and stimulated conditions. The values are expressed in the range of 0 to 1, with 1 denoting perfect circularity. Statistical significance was determined using ANOVA and Student’s t-test (* and * P<0.05).* denotes statistically significant differences between transfected and untransfected cells for each of the two conditions (thrombin stimulated and unstimulated cells) * denotes statistically significant differences between thrombin stimulated and unstimulated cells for each condition.
5.9.3 Differential effect of calpain isoforms on endothelial resistance to shear stress

The effect of calpains the distribution and size of focal adhesions suggested that calpains could be involved in regulating the endothelial monolayer's response to shear stress. By 60 min, most control cells had detached with of 12 dyn/cm² shear stress (Figure 5.23).

The response of calpain-1 siRNA-treated EA.hy926 cells closely resembled that of mock transfected EA.hy926 cells. 50% and 35% of cell detached in siGLO and calpain-1 siRNA-treated cells respectively, after 45 min of exposure to flow. By 60 min of shear stress application very few cells had remained attached to the substrate: 30% and 35% in mock and calpain-1 siRNA-transfected EA.hy926 cells. In contrast, greater resistance to flow was exhibited by RNAi-induced down-regulation of the calpain-2 isoform, with a maximum of 75% remaining attached after 60 min of shear stress application.

Calpain-2 therefore appeared to have an important role in the shear stress pathway. Inhibition of focal adhesion disassembly as a result of reduced expression of the calpain-2 isoform could provide the sufficient strengthening of cell attachment to the substrate thereby increasing endothelial resistance to shear stress.
Confluent EA.hy926 cells were transfected with calpain-1 or calpain-2 siRNA together with the red transfected indicator siGLO. After 48 hours, siRNA-treated and cells transfected with siGLO alone, were exposed to 12 dyn/cm shear stress. Cells were visualized by time-lapse microscopy using an Eclipse TE250-E Microscope and a X20 air objective (see). The figure includes only the time-points when cell detachment occurred as a result of shear stress. Frames of the red indicator are not shown as the transfection efficiency of the indicator was > 95%. The black arrow indicates flow direction. The graph shows the number of cells adherent to the cell surface for each time point expressed as a percentage of the number of cells at t=0. Representative of two experiments, bar= 20 μm.
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5.10 Discussion

The role of calpain in various signalling pathways has been the subject of extensive studies which often involve overexpression of the endogenous calpain inhibitor calpastatin and the effect of chemical inhibitors of which calpeptin and calpain inhibitor-I (ALLN) have been used the most. The generation of the chemical inhibitors involves modifications of a plant-derived inhibitor, namely leupeptin, aimed at improving the inhibitors' cell permeability as well as their specificity to calpain (Donkor, 2000). To date, nothing has been published as to the effects of these inhibitors or the role of calpain in EA.hy926 cells.

5.10.1 Calpain inhibitor specificity

While both calpeptin and calpain inhibitor-I exhibit increased cell permeability compared to leupeptin and both are able to inhibit calpain, neither is a specific inhibitor to calpain. Instead both inhibitors have been reported to be better inhibitors of other neutral proteases such as cathepsin family proteases (Donkor, 2000).

The unspecific function of the two inhibitors was clearly demonstrated by comparing and contrasting the effects induced in EA.hy926 cells as a result of treatment with calpeptin, calpain inhibitor-I or both. Although differential responses to the various treatments were expected, the differences strongly indicate that the inhibitors do not specifically inhibit calpain. Lack of specificity of calpeptin has also been reported by Schoenwaelder and Burridge (1998) based on observations that treatment of fibroblast cells with the inhibitor resulted in morphological changes that were not observed in treatments with other calpain inhibitors.

Hence, any conclusions regarding the function of calpain should not be based solely on the effects induced by calpain inhibitors. As the RNAi-induced down-regulation of calpain-1 and calpain-2 together led to the detachment of EA.hy926 cells, it was not possible to use this approach to verify observations with the inhibitors.
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The results obtained by different calpain inhibitor treatments in EA.hy926 cells will therefore be compared and contrasted to already published data investigating the role of calpain in order to deduce which of the changes observed could be attributed to specific inhibition of calpain.

5.10.2 Calpain and the actin cytoskeleton
Gonscherowski et al. (2005) reported rapid rounding and cell retraction of cultured HUVECs in response to treatment with calpain inhibitor-I and confirmed it by treating cells with a membrane-permeable conjugate of calpastatin peptide-penetratin (CPP). The endothelial cell contraction was reported to be associated with increasing translocation of Rac and Rho GTPases to the cell membrane signifying activation of these proteins upon inhibition of calpain.

Similarly, rapid cell rounding in EA.hy926 cells occurred within seconds of simultaneous incubation of the cells with both inhibitors. An increase in RhoA activity was not confirmed in EA.hy926 cells but increased levels of Rac1 GTP were observed in the presence of calpain inhibitors under unstimulated conditions, indicating activation of the Rho GTPase. Although cell rounding was also observed in EA.hy926 cells treated with calpain inhibitor-I alone, this was to a lesser extent than in EA.hy926 cells and its association with Rac1 activity levels was not determined. Most intense contraction of EA.hy926 cells occurred in response to calpeptin. Even though the calpain inhibitors affected the cells to different degrees, endothelial cell rounding occurred in response to all three conditions and agreed with results obtained from CPP (Gonscherowski et al. 2005). Though not definitive, it is possible that endothelial cell rounding results from specific inhibition of calpain.

RNAi-induced down-regulation of calpain-1 and calpain-2 isoforms led to cell rounding and cell spreading in unstimulated EA.hy926 cells respectively, thereby demonstrating that depending on which calpain isoform is inhibited different effects are exerted in endothelial cells. If the inhibitor-induced effects were in fact the result of specific
inhibition of calpain, the differences could be explained by greater inhibition of calpain-1 and calpain-2 by calpeptin and calpain inhibitor-I respectively.

5.10.3 Calpain and focal adhesions
Huttenlocher et al (1997) demonstrated a decrease in the size and numbers of focal adhesions in response to calpain inhibitor-I treatment, resulting in increasing adhesiveness and decreased rate of cell detachment in migrating cells. The results were confirmed by the group using other calpain inhibitors (not calpeptin), namely calpain inhibitor-II and BDK. They also demonstrated that the calpain responsible for the above effects was calpain-1 via characterization of migration in CHO cells expressing low levels of calpain-1 and CHO cells containing 10% calpain I and 90% calpain II.

Similarly, simultaneous incubation of EA.hy926 cells with calpeptin and calpain inhibitor-I resulted in the generation of larger focal adhesions located at the cell periphery which was associated with a reduction in numbers. The effect was stronger by treatment of EA.hy926 cells with calpeptin alone, whereas in response to calpain inhibitor-I focal contact characteristics were similar to those of untreated control cells.

Observations based on RNAi induced down-regulation of calpain isoform expression, revealed that in contrast to CHO cells, calpain-2 was the isoform responsible for the generation of bigger and fewer focal adhesions. In agreement to the conclusions drawn by Huttenlocher et al. (1997) increased adhesiveness and decreased rate of cell detachment was observed as a result of bigger focal adhesions as illustrated by increased resistance of calpain-2 siRNA treated EA.hy926 cells to shear stress.

As in the case of endothelial cell contraction, in addition to unspecific inhibition, the differential effects of calpain inhibitors could be explained by greater inhibition of calpain-1 and calpain-2 by calpeptin and calpain inhibitor-I respectively.

The presence of big focal adhesions correlates calpain function to the disassembly of focal adhesions and hence the regulation of focal adhesion turnover. Although the role of
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calpain in focal adhesion disassembly is isoform-specific, which of the two calpains plays this role appears to could depend upon cell type.

5.10.4 Calpain induced proteolysis of focal adhesion proteins

Several publications implicate calpain-mediated proteolysis of focal adhesion proteins as the mechanism by which calpains are able to regulate focal adhesion turnover. Amongst the reported calpain substrates are FAK, Src and talin, for which calpain mediated proteolysis has been demonstrated using calpain inhibitors, calpastatin over expression or focal adhesion protein point mutations at calpain cleavage sites rendering them resistant to proteolysis by calpain (Cooray et al., 1996; Oda et al., 1993; Franco and Huttenlocher, 2005; Beckerle et al., 1987).

In platelets, calpeptin has been reported to: prevent calpain-induced proteolysis of focal adhesion proteins (FAK and talin) and phosphatases (PTP-1B) after Von Willerbrand Factor (VWF)-dependent translocation to the cytoskeleton, induce a dramatic decrease in the activity of FAK and its dissociation from the cytoskeletal fraction (Coorray e.t al., 1996) and prevent thrombin/calcium ionophore-induced proteolysis of Src (Oda et al., 2003). Calpain-induced FAK proteolysis has also been demonstrated in fibroblasts via overexpression of calpastatin. Carragher et al. (2001) demonstrated prevention of FAK proteolytic cleavage resulting in impaired disassembly of focal adhesions and a lack of progression of vSrc-transformed cells through G1 phase.

As neither FAK nor Src proteolysis was observed in response to thrombin or shear stress and due the technical difficulties associated with attempts to follow talin proteolysis (data not shown) no deductions can be made regarding calpain-mediated proteolysis of focal adhesion proteins in EA.hy926 cells. In hindsight it would have been interesting to have observed the effect of calcium ionophore (positive regulator of calpain activity) on the proteolysis of FAK and Src in the presence of calpain inhibitors as well as in calpain-1 and calpain-2 siRNA treated EA.hy926 cells as calpain-induced proteolysis has not been investigated in endothelial cells to date.
5.10.5 Calpain and the microtubule cytoskeleton

Inhibition of microtubule-mediated turnover of adhesive complex sites by calpain inhibitor-I or calpeptin occurs after nocodazole wash-out suggesting calpain-mediated regulation of focal complex disassembly downstream of microtubules (Bhatt et al., 2002). They reported that the prevention of calpain-mediated focal adhesion disassembly in fibroblasts by calpain inhibitor-I was not the result of microtubule disruption. In fact, neither microtubule disruption nor tubulin de-stabilization has been reported as a consequence of calpain inhibition.

In agreement, microtubule disruption did not occur in response to calpain inhibitors in EA.hy926 cells. Confirmation was offered by the RNAi experiments indicating that neither calpain-1 nor calpain-2 led to microtubule disruption.

As suggested previously, decreases in acetylated tubulin as a result of calpeptin treatment (or treatment with both inhibitors) could be resulting from inhibition of proteases other than calpain. It is possible that severing of microtubules (see Chapter 1.8.3) occurs, enhancing tubulin deacetylation until cells adapt morphologically, at which stage microtubules are able to polymerize again and become more stable. Cell blebbing could be the consequence of inhibitor-induced cell rounding. High resolution time lapse microscopy could be used in future experiments to determine whether cell blebbing occurs in calpain inhibitor-treated EA.hy926 cells.

5.10.6 Calpain and tyrosine phosphorylation

In addition to protein proteolysis, it has been suggested that calpain regulates focal adhesion turnover by modulating the phosphorylation state of focal adhesion proteins.

Calpeptin-induced inhibition of tyrosine phosphatases and consequent enhancement of tyrosine phosphorylation, particularly of paxillin was reported by Schoenwaelder and Burridge (1998). As mentioned in chapter 5.1.10, the investigation compared the effects of calpeptin to those of other calpain inhibitors including calpain inhibitor-I (ALLN) and found them to differ with respect to stress fiber formation and tyrosine phosphorylation;
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the results suggested that stress fiber formation could have been promoted by calpeptin-induced inhibition of protein tyrosine phosphatases (PTPs) via binding of calpeptin to the critical cysteine residue in the active site of PTPs (preferentially membrane associated PTPs). Calpeptin could not induce stress fibers in Rho-inhibitor-treated cells, implying that membrane associated PTPs act upstream of Rho. Calpeptin could therefore lead to an increase in RhoA-activation and explain the enhanced phosphorylation of MLC observed in the current investigation.

Nevertheless, the role of calpain in regulating phosphorylation of tyrosine residues has been reported based on the effects of active calpain rather than on cell responses to its inhibition. In platelets, active calpain induces alterations in the balance between tyrosine kinase and phosphatase activities resulting in a net decrease in phosphotyrosine levels (Pain et al., 1999). A similar regulatory function of calpains has also been reported in neuronal cells, where calpain-mediated localized reduction in tyrosine phosphorylation at filopodia proved to be sufficient for mediating their stabilization (Robles et al., 2003).

Increased phosphorylation of Y397-FAK, Y118-paxillin and Y31-paxillin were exhibited in EA.hy926 cells treated with both calpain inhibitors under unstimulated conditions. As discussed in Chapter 5.5.1, these changes were consistent but small, which meant that they were not statistically significant, but could be explained by the suggested function of calpain as a modulator of phosphatase and kinase activities (Pain et al., 1999; and Robles et al., 2003). The activity of Src depends on the phosphorylation of Y416 and Y527, which are responsible for the activation and inactivation of Src kinase activity respectively (see Pertone and Sap, 2000). The decreased levels of pY416-Src as a result of calpain inhibition could be justified by calpain-inhibitor-induced increases in pY527-Src levels. Whether the phosphorylation status of the two residues is linked is not yet known.

Calpain inhibitor treatment in EA.hy926 cells was observed to inhibit the thrombin-induced phosphorylation changes, indicating that calpains regulate the thrombin response. Based on the effects of RNAi treatment on the thrombin response, it was
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deduced that calpains are indeed involved in the thrombin signalling pathway. Experimental results suggested that calpain-2 rather than calpain-1 is responsible for regulating the thrombin response, as inhibition of thrombin-induced endothelial cell contraction was observed only in cells with decreased expression of calpain-2 and hence focal adhesion stabilization. The inhibition of thrombin-induced phosphorylation by calpain inhibitors implies that calpain-2 could be responsible for inducing these phosphorylation events in response to thrombin, a conclusion that contradicts the proposed role of active calpain in inducing a net decrease in tyrosine phosphorylation (Pain et al., 1999; Robles et al., 2003).

Nevertheless, the above observations do not necessarily indicate a direct regulation of tyrosine phosphorylation by calpain action; so far, the thrombin-induced morphological changes have been viewed as a consequence of the changes in tyrosine phosphorylation of focal adhesion proteins. It is possible however, that the reverse is true and that changes in protein phosphorylation are a consequence of rather than causing the action of thrombin-induced changes in cell morphology. Hence, the inhibition of thrombin-induced phosphorylation of FAK, Src and paxillin could therefore be the result of the calpain-2-mediated inhibition of thrombin-induced endothelial cell contraction rather than its consequence. This hypothesis offers explanations of the observations in EA.hy926 cells without contradicting the suggested function of calpains in mediating decreases in tyrosine phosphorylation.

Despite the dependence of the thrombin response on calpain-2, under unstimulated conditions inhibition of both calpain-1 and calpain-2 was observed to regulate cell shape, by inducing cell contraction and spreading respectively. Future investigations of the effects of knocking down each of the two calpain isoforms inhibitors on tyrosine phosphorylation of FAK, Src and paxillin could therefore provide some insight in the connection between cell morphology and tyrosine phosphorylation of focal adhesion proteins. In addition to having different functions, the activity of the two calpains could be under localized regulation.
As comparisons between RNAi and inhibitor effects in EA.hy926 cells have suggested preferential calpain-1 and calpain-2 inhibition by calpeptin and calpain inhibitor-I respectively, it would be interesting to observe whether a similar preference exists with respect to protein tyrosine phosphorylation. Another interesting aspect of protein tyrosine phosphorylation that could be worth pursuing further in investigations is whether in addition to inhibiting calpain-mediated proteolysis of focal adhesion proteins, inhibition of calpain-mediated reduction in tyrosine phosphorylation of focal adhesion proteins could be contributing to the generation of larger focal adhesions.

Preferential activation of calpain-2 rather than calpain-1 has also been reported in EGF-induced responses in fibroblasts (Shao et al., 2006). Shao et al. (2006) reported the EGF-induced localized activation of m-calpain/calpain-2 at the rear of locomoting fibroblasts required for de-adhesion of the cell body, enabling cell movement. The group aimed to explain the direct activation of calpain-2 by ERK, a process that was previously reported essential for EGFR-induced motility. As the calcium requirement for calpain-2 activation was higher than the localized calcium mobilization caused by EGF, the group suggested that binding of phospholipids to calpain-2 could be involved in reducing the calcium requirement for calpain-2 activation. Previous findings had indicated that PIP$_2$ is preferentially hydrolysed at the leading edge of migrating cells and a decreasing gradient of PIP$_2$ from cell body to the lamellipodium. By hypothesizing that phospholipids play a role in restricting calpain-2 activity to non-lamellipodial regions the group was able to demonstrate the activation and co-localization of calpain-2 with PIP$_2$ and phospholipase-C (PLC) at the rear membrane of a moving cell in response to EGF stimulation. By combining the data, the group suggested a model of calpain-2 binding to PIP$_2$ concurrent with and likely to enable ERK activation providing a mechanism by which cell de-adhesion is directed to the cell body and tail as phospholipase C hydrolyzes PIP$_2$ in the protruding lamellipodia. The findings of Shao et al. (2006) therefore demonstrate that the two calpain isoforms are able to be differentially regulated not only in time but also in space.
5.10.7 Conclusions
Calpain therefore appears to be playing an important role in regulating focal adhesion disassembly in EA.hy926 cells. In contrast to platelets for which calpain-1 has been reported to be the main regulator of focal adhesion turnover, in EA.hy926 cells calpain-2 was observed to hold a more central role to calpain-1, both in thrombin and shear stress responses.
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The aim of this investigation was to contribute to the understanding of the mechanisms by which endothelial cells respond to thrombin and shear stress, as well as offering comparisons between the effects of the two stimuli and revealing cell type specificity and signal magnitude dependence. Although the morphological responses differed between shear stress and thrombin, both stimuli were able to regulate the phosphorylation of focal adhesion proteins in similar manners. The results obtained from this study are illustrated in figure 6.1.

The effects of thrombin on several types of endothelial cells have been described extensively, but limited investigation has to date demonstrated those effects on the endothelial cell line EA.hy926. On the whole, EA.hy926 cells responded to thrombin in similar ways to other endothelial cells including increased cell contraction, fragmentation of VE-cadherin staining, increased cortical stress fibre content, increased phosphorylation of focal adhesion proteins, increased RhoA activity and no change in Rac1 activity. Morphological changes induced by thrombin occurred very early and coincided with increases in RhoA activity, whereas the phosphorylation status of focal adhesion proteins was not altered until later time-points. Hence, thrombin-induced morphological changes are likely to be due to signalling events directly downstream the thrombin receptor (PAR) rather than integrins. Regulation of the phosphorylation status of focal adhesion proteins could therefore be the consequence of “inside-out” signalling due to thrombin-induced actin remodelling or the result of PAR-1 mediated integrin activation. In the current investigation thrombin was used as 2300 nM (Chapter 2.2.1.2) while the physiological maximum for thrombin in the procoagulant response has been reported to be around 1500 nM (Chapter 1.4.2). Since EA.hy926 cells have been observed to respond to lower concentrations of thrombin (figure 3.1) it would be interesting and important in terms of physiological relevance to investigate the effects of lower thrombin concentrations on the overall response of EA.hy926 cells.
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Under physiological conditions, veins and arteries are exposed to shear stress magnitudes in the range of 1 to 6 dyn/cm$^2$ and 10 to 70 dyn/cm$^2$, respectively. In this study, exposure of EA.hy926 cells representing veins and BAECs representing arteries to low magnitudes within the physiological range induced rapid phosphorylation changes in focal adhesion proteins. Thus, it was hypothesized that unlike thrombin, shear stress was able to stimulate integrin receptors directly. However, activation of receptors other than integrins such as PECAM-1, calcium channels and VEGF receptor has been reported in response to shear stress (Kwan et al., 2003; Rensik et al., 2003). In order therefore to determine the mechanism by which thrombin or shear stress affect the morphology of endothelial cells, the activity of integrins, PAR-1 and several other receptors in response to stimulation should be investigated. Under physiological stress the two cell types used as models for the response in veins (HUVECs and EA.hy926 cells) responded in different ways both morphologically as well as biochemically. Interestingly when HUVECs were exposed to higher than physiological shear stress levels for veins (12 dyn/cm$^2$), the response shared greater similarities to that of EA.hy926 cells exposed to shear stress levels within the physiological range (1.6 dyn/cm$^2$). The HUVEC response to low shear stress suggested that signalling does not necessarily occur via the regulation of focal adhesion proteins and may possibly not necessarily require the activation of integrin receptors. Even though it is possible that the observed differences resulted due to cell type specificity, it could be that different receptors are activated in each cell type at low shear stress. Determining which receptors are activated would provide insights as to the different mechanisms associated with each response.

As mentioned before, increases in cortical actin filaments as a result of thrombin stimulation involved the activation of RhoA yet did not induce MLC phosphorylation. Thus it was suggested that the observed contraction could be a consequence of passive cell retraction (reviewed in Bogatcheva et al., 2001). In the shear stress response on the other hand, increases in stress fibre content spanning the cells was associated with MLC phosphorylation. It is nevertheless possible that MLC phosphorylation is required for the thrombin-induced cell contraction and that this occurs much earlier in the time course. Based on the results of this investigation, no firm conclusions can be drawn regarding the
mechanism by which actin re-organization is induced in response to either thrombin or shear stress in endothelial cells. The antibody used in this investigation to identify phosphorylated MLC was one that recognized phosphorylated Thr18 and Ser19 residues. The serine residue is the major phosphorylation site of MLC. Phosphorylation of the threonine residue occurs at a slower rate under conditions of maximum stimulation (Burridge and Chrazowska, 1996). As discussed previously, ROCK, a downstream target of RhoA, is also able to phosphorylate Ser19-MLC. Antibodies recognizing pSer19-MLC could therefore be used to determine whether ROCK-mediated MLC phosphorylation occurs in the pathways under investigation (Totsukawa et al., 2003).

Amongst the cytoskeletal regulators whose phosphorylation status should be studied under the conditions investigated is LIMK-1, a downstream effector of ROCK. The endothelial responses that induced stress fibre formation were also linked to tubulin deacetylation (legend of figure 6.1). Accumulating evidence suggesting a cross talk between actin and microtubules based on which a model has been proposed regarding cytoskeletal regulation in the response to thrombin, which involves the activation of LIMK-1 (Figure 6.2). Nevertheless, this model remains to be proven or refuted, while the mechanism by which dissociation of LIMK-1 from the microtubules leads to tubulin deacetylation is still uncertain. As illustrated in the Figure 6.2, ROCK activity is regulated by RhoA. It is therefore possible that this model holds for the thrombin response, as EA.hy926 cells exhibited increased levels of RhoA activity upon stimulation. As discussed previously, investigating the effect of shear stress on the activity of RhoA would provide insights as to the differences associated with increasing formation of stress fibres between the thrombin and shear stress responses. RhoA GTP is only best-studied mediator of MLC phosphorylation but other small Rho GTPases have also been implicated in the regulation of MLC, including Cdc42 and Rac1 (Yang et al., 2006; Pestonjamsap et al., 2006). Investigating the effect of shear stress on the activity of RhoA, Rac1 and Cdc42 would allow for a better elucidation of the mechanisms involved in the different responses.
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Stress fibre formation and tubulin de-acetylation occurred prior to regulation of focal adhesion proteins in the thrombin response, but after with shear stress (figure 6.1). Based on the timing alone, a dependence of the cytoskeletal changes on focal adhesion protein phosphorylation or vice versa can not be proven. In order to deduce the order of events, cytoskeleton stabilizing and de-stabilizing agents could be used to help distinguish between events occurring downstream or upstream of the cytoskeleton. Investigating the effect of thrombin and shear stress on intermediate filaments and especially vimentin, which is reorganized in response to shear stress (Helmke et al., 2001) and phosphorylated in response to thrombin (Bormann et al., 1986) would further contribute to the understanding of how the two stimuli affect the endothelial cytoskeleton.

An overall limitation for the comparison of HUVEC with EA.hy926 cell responses was the presence of fibronectin substrate for experiments involving the former but not the latter. Via strengthening adhesiveness, fibronectin could be affecting integrin-mediated signalling. The comparison, though, could have been compromised even if fibronectin was used as an EA.hy926 cell substrate; in contrast to HUVECs, fibronectin was not essential for the adhesion of the cell line, to any surface, implying that their adhesiveness is probably greater than that of HUVECs and addition of substrate could contribute in further enhancing the adhesion thereby affecting integrin-mediated signalling. The difference in adhesiveness is important especially with respect to shear stress experiments where poorly adherent cells could have fewer contacts and hence less shear stress induced signalling. This, in addition to reasons discussed above, could also explain the differences between HUVEC and EA.hy926 cell responses to low shear stress. When studying the effect of shear stress in the present system, in which cells increasingly detached with time (especially from the centre of the monolayer) it is also important to consider the possibility that the loss of these cells changes the properties of the cells that remain attached. For example, the few remaining cells could flatten out and become more firmly adherent.

Currently the proposed model in figure 6.1 is incomplete. The construction of the diagram was based on the observed timing of events taking place but for a more complete
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and accurate account further experiments are required. Focal adhesion protein-protein interactions through immunoprecipitation experiments should be investigated. Since changes in the phosphorylation of different proteins occurred at similar time-points, determining interactions between the focal adhesion proteins could help deduce the sequence of events. Complementing these results with immunofluorescence experiments such as with immunolocalization of pY576-FAK or pY31-paxillin would allow localized phosphorylation changes to be detected. Identifying the phosphatase responsible for the de-phosphorylation of Y31-paxillin and observing whether other focal adhesion proteins are substrates of the phosphatase would constitute an interesting addition to the model. Furthermore, automated quantification of the number and size of focal adhesions, using for example software such as MATLAB, would help determine whether formation of new focal adhesions or translocation of proteins to focal adhesions takes place in response to shear stress or thrombin similar to what has been shown in fibroblast cells using different techniques (Ballestrem et al., 2006; Spatz and Geiger, 2007). Similarly quantifying the amount of stress fibres as a result of stimulation would help distinguish between actin re-organization and actin polymerization. Focal contact and stress fibre quantifications should also complement results on the effect of calpain inhibition. Quantification of results would provide a more accurate basis for drawing conclusions.

As the investigation using calpain inhibitors shows, additional data are required to determine the role of calpains in EA.hy926 cells. It is important that the results take into account the implication of unspecific inhibition such as inhibition of papain or cathepsin mediated by calpeptin and calpain inhibitor-I respectively. Overexpression of the endogenous calpain inhibitor calpastatin or the use of calpain knock-out endothelial cells would help distinguish between observations resulting from inhibition of calpain versus other proteases.

Calpain inhibitors do not distinguish between the roles of calpain isoforms. No isoform preference of the inhibitors has so far been reported. In this study, comparisons between the siRNA derived data with data obtained from calpain inhibitors, revealed certain similarities between calpeptin-treated cells and cells with siRNA-reduced calpain-1 as
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well as between calpeptin inhibitor-I-treated cells and cells with siRNA-reduced calpain-2 expression.

Studying the effect of calpain knock down on the phosphorylation of focal adhesion proteins would help confirm whether the increases observed upon calpain inhibitor treatment are the consequence of specific inhibition of calpain.

Calpains, however, do not only alter phosphorylation of focal adhesion proteins. The function of calpains as proteases cleaving focal adhesion proteins has been reported in various cell types. It is possible that calpain-mediated proteolysis of talin, FAK or Src occurs in response to both thrombin and shear stress, though not observed in this investigation (not shown). Published data regarding talin proteolysis suggest that separation of full length talin from its cleaved product can be achieved with the use of high salt concentration in NP40 lysis buffer separation of full length talin (Franco et al., 2004). Further experiments therefore should include the use of different lysis buffers in order to observe whether the calpain-mediated proteolysis of focal adhesion proteins occurs in EA.hy926 cells in response to thrombin and shear stress.

As described in materials and methods, stable fluorescent non-targeting control siRNA (siGLO-green or red) were used in experiments in order to help in the identification of transfected EA.hy926 cells. However, the apparent high (95%) transfection efficiency indicated by the siGLO, does not necessarily indicate efficient knock down of the protein under investigation, calpain in this case. Preliminary immunofluorescence experiments using calpain-isoform specific antibodies (data not shown) suggested they could be used to identify cells knocked down by calpain expression.

In the course of this study, greater difficulties in obtaining immunofluorescence data were encountered in shear stress experiments. Due to the quality of staining obtained on the plastic shear stress chambers the generated data regarding the localization of proteins was limited. Nevertheless, this can be overcome with the emergence of newer and better quality chambers such as those used in the shear stress system 2. Unfortunately the
system was obtained later in the progress of this study and was not used to the maximum of its potential. The lack of data on the effect of shear stress on BAEC morphology was due to limited availability of these cells. Such investigation would have provided insights as to the relationship between biochemical and morphological changes and a better comparison between responses of different cell types.

In this study, the effect of laminar shear stress within the physiological range of veins and arteries was investigated. Shear stress of the type and magnitude used in this investigation is thought to be atheroprotective. In contrast oscillatory (turbulent) flow is thought to be atherosclerotic. Comparison between the effects of "atheroprotective" and "atherosclerotic" shear stress on endothelial cells could provide insights in the differences between the two mechanisms. Technological advancements can also help in establishing greater biological significance; endothelial cells can be cultured in shear stress chambers mimicking the cylindrical nature of blood vessels or arterial bifurcations. As discussed previously, both shear stress and thrombin have been implicated at the initial stages of atherosclerosis (Davies et al., 1995; Lentz et al., 2002; Ming et al., 2004). Once studied independently, simultaneous exposure of endothelial cells to shear stress and decreasing thrombin concentrations could help in the understanding of how this occurs in vivo.

It is nevertheless important that care is taken when interpreting in vitro experiments with respect to physiological relevance and more so when data is generated based on observations using cell lines such as EA.hy926. As discussed in chapter 1.11, comparative studies between primary cells (HUVEC) and the EA.hy926 cell line have identified differences in gene expression that compromise the endothelial nature of the cells and thereby the extend to which generated data can be assumed to be representative of primary cells. As for all in vitro experiments, whatever the design aiming to resemble the physiological environment, in vivo experiments are required for a better understanding of the physiological processes.


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EA.hy926
1.6 dyn/cm²

HUVEC
1.6 dyn/cm²

HUVEC/BAEC
12 dyn/cm²

EA.hy926
12 dyn/cm²

Thrombin

Y31

Phosphatase

P

Rho-GTPases?
MLCK?

P

Active calpains
induce focal adhesion turnover. Inhibition: prevents dissociation of proteins from complex increasing stability thereby increasing spreading and enhancing cell attachment to the substratum.

PAR with amino terminus exposed

Integrin Receptors

G-protein

Thrombin pathway

Shear stress Pathways

Inhibited by calpain inhibition

Talin

pY118-paxillin

pY397,Y576-FAK

pY416 Src

IAP

0

Thrombin with amino terminus exposed

Integrin Receptors

G-protein
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**Figure 6.1: A hypothetical model:** Shear stress directly activates integrins and the subsequent formation of focal adhesion complexes with alterations in the phosphorylation status of focal adhesion proteins paxillin, FAK and Src. The exception is HUVEC exposed to low shear stress under which the phosphorylation of paxillin only is affected. Unlike other EA.hy926 cells low shear stress induces the formation of stress fibres in HUVECs probably via the phosphorylation of MLC. Thrombin activates PAR receptors leading to cell rounding, reorganization of stress fibres and an increase of cortical actin through activation of RhoA. Activation of integrins and formation of focal adhesion complexes with alterations in the phosphorylation status of focal adhesion proteins could be occur at a later stage via “inside-out” signalling following the formation of stress fibres and/or via indirect interaction between activated PARs and integrins (via IAPs). Intracellular calcium release occurs within seconds of thrombin stimulation leading to the activation of calpains (mainly calpain-2) involved in the regulation of focal adhesion turn-over, which could be required for efficient cell rounding in response to thrombin. The function of calpain-2 as regulator of focal adhesion turnover could also be important for endothelial cell resistance to shear stress suggested by observations in EA.hy926 cell exposed to high shear stress. Stress fibre formation, whether in response to thrombin or shear stress, occurs in parallel to tubulin deacetylation. The association between stress fibre formation and microtubule destabilization could be regulated by calpain, since depending on the means of inhibition, varying degrees of stress fibre formation and tubulin deacetylation were observed.
Figure 6.2: A proposed model of how LIMK1 regulates the actin cytoskeleton and microtubules. The figure was taken from Gorovoy et al. (2005), and suggests that in unstimulated endothelial cells LIMK-1 is associated with microtubules and disassociates when phosphorylated/activated by ROCK. As a result, microtubule de-polymerization is induced and phosphorylated LIMK-1 is released into the cytoplasm phosphorylating cofilin thereby leading to an increase in polymerized actin.
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