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β1- integrin: An endothelial mechanoreceptor distinguishing force direction

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To my second mum, Lena, who will always be by my side

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Summary

Endothelial cell (EC) function is affected by forces generated by flowing blood on the arterial wall (wall shear stress, WSS). Unidirectional WSS and laminar flow occurs in unbranched areas of the arteries, which are protected from atherosclerosis, whereas oscillatory WSS and “disturbed flow” occurs at branch points, where the majority of atherosclerotic plaques are initiated. EC can sense forces via multiple mechanoreceptors including β1- integrin, which induces downstream signalling in response to WSS. Although EC can distinguish between unidirectional and oscillatory WSS, the mechanisms that control this are unknown.

We hypothesized that mechanoreceptors are activated by specific mechanical conditions which trigger downstream signalling, i.e. some mechanoreceptors are activated by unidirectional WSS whereas others are activated by bidirectional force. We examined the effect of flow direction and frequency of oscillation on calcium signalling and how the mechanical activation of β1- integrin contributes to these responses.

We found significant differences in calcium dynamics in ECs exposed to different flow conditions. Using magnetic beads, unidirectional force but not bidirectional force applied directly to apical β1- integrin induced its conversion from a bent inactive to an extended active form. Furthermore, application of unidirectional force to β1- integrin induced calcium release from the inner stores of the endoplasmic reticulum. Finally, studies using pharmacological inhibitors revealed that phospholipase C and ROCK regulate the calcium responses triggered by mechanostimulation of β1- integrin.

These results are consistent with the hypothesis that distinct endothelial mechanoreceptors respond to different flow patterns. My work suggests that β1- integrin functions as a mechanoreceptor that can distinguish between different force directions.

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Abbreviations

AFM	atomic force microscopy
apoE	apolipoprotein E
BMPR	bone morphogenic protein receptor
BSA	bovine serum albumin
Ca	calcium
CAM	cell adhesion molecules
cDNA	complimentary DNA
cm	centimeter
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
EC	endothelial cell
ECM	extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
endoMT	endothelial to mesenchymal transition
eNOS	nitric oxide synthase 3
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinases
FGFR	fibroblast growth factor receptor
FRET	fluorescence resonance energy transfer
GAGs	glycosaminoglycans
GPCR	GTP- binding protein- coupled receptor
GTP	guanosine-5'-triphosphate
HCAEC	human coronary artery endothelial cell
HSS	high shear stress
HPRT	hypoxanthine guanine phosphoribosyltransferase
HUVECs	human umbilical vein endothelial cells

Hz	Hertz
IMS	industrial methylated spirit
JNK	Jun amino-terminal kinases
KLF-2	kruppel- like factor 2
LDL	low density lipoprotein
LSS	low shear stress
MAPK	mitogen activated protein kinase
MMPs	matrix metalloproteinases
NF- κB	nuclear factor kappa- B
NO	nitric oxide
P2X4	P2X purinoceptor 4
p53	tumour protein p53
PBS	phosphate buffered saline
PECAM- 1	platelet endothelial cell adhesion molecule 1
PI3 kinase	phosphatidylinositol- 3- OH- kinase
pN	picoNewtons
PIP2	phosphatidylinositol- 4,5- biphosphate
PKCζ	protein kinase C, zeta
PLCi	phospholipase C inhibitor
qRT- PCR	quantitative real time polymerase chain reaction
RCF	relative centrifugal force
RGD	Arg-Gly-Asp
ROCK	Rho-associated protein kinase
RTK	receptor- tyrosine kinase
SBS	sodium buffered saline
SEM	standard error of the mean
SERCA	sarco/endoplasmic reticulum calcium ATPase
siRNA	small interfering RNA
SOCE	store operated calcium entry

TAWSS	time- average wall shear stress
TNF- α	tumour necrosis factor α
tpsg	thapsigargin
transWSS	transverse wall shear stress
TRP	transient receptor potential cation channel
VCAM-1	vascular adhesion molecule 1
VE- cadherin	vascular endothelial cell cadherin
VEGFR2	vascular endothelial growth factor receptor 2
VSMC	vascular smooth muscle cells
WSS	wall shear stress

Chapter 1: Introduction

1.1 Atherosclerosis

Atherosclerosis is a lipid-driven chronic inflammatory disease that is the leading cause of morbidity and mortality in the developed world. It is the most common cause of large vessel pathology promoting the formation of atherosclerotic plaques, which block the normal blood flow, leading to disease such as stroke and myocardial infarction (Ross, 1993, Ross, 1999, Hansson, 2005). As atherosclerosis develops, inflammatory cells, extracellular matrix (ECM) and lipids penetrate into the arterial wall leading to the formation of plaques. These plaques reduce and potentially block normal blood flow through arteries. Although lifestyle changes would delay the progression of cardiovascular disease, gaining deeper knowledge of the genetic and molecular determinants may provide the opportunities to develop novel preventative and treatment techniques.

Endothelial activation and inflammation contributes to the initiation and progression of atherosclerosis. Slow and disturbed blood flow in specific areas of the vasculature can induce endothelial activation via induction of adhesion molecules, such as P- selectin, E- selectin and vascular cell adhesion molecule- 1 (VCAM-1) leading to attachment of inflammatory cells (Olgac et al., 2009, Koskinas et al., 2010). In the beginning, they attach loosely and then firmly. After attachment, monocytes penetrate into the sub- endothelial space. Subsequently, the monocytes transform into macrophages (Galkina and Ley, 2009). This process relies on oxidative modification of circulating low- density lipoprotein (LDL) by reactive oxygen species and their ingestion by macrophages, which can subsequently form lipid- laden foam cells.

The process of atherogenesis continues with the progression of the atherosclerotic plaque, which has a characteristic architecture. Atherosclerotic plaques can be either stable or unstable (vulnerable). However, atherosclerotic plaques exhibit plasticity in their stable versus unstable characteristics. In fact, they can shift between the two conditions depending on the micro- environment (van der Wal and Becker, 1999).

Stable atherosclerotic plaques are rich in smooth muscle cells and extracellular matrix and they tend to be asymptomatic. On the contrary, unstable plaques are rich in foam cells and macrophages. Unstable plaques have a fibrous cap that is prone to rupture (Finn et al., 2010). The rupture of the fibrous cap can lead to formation of a thrombus in the lumen. Occasionally, these thrombi detach from the lumen and through the circulation move and block downstream branches, leading to thromboembolism. (Falk, 1983, Rauch et al., 2001, Libby and Simon, 2001).

In conclusion, atherogenesis is a long- standing process that involves several molecular signaling cascades. A better understanding of events prior to the activation of cells relative to the disease, would give us the ability to interfere and prevent the progression of the disease.

1.2 Blood flow patterns and atherosclerosis

There are a variety of significant risk factors such as obesity, smoking, age and high cholesterol that raise the risk of plaque formation. However, plaque formation occurs at specific areas of the vasculature. The majority of atherosclerotic plaques are found at branch points and arterial bifurcations. On the contrary, plaques are not often detected at straight unbranching areas of the arteries (Ku et al., 1985).

The luminal side of blood vessels is lined by a thin monolayer of cells, called the vascular endothelium. The vascular endothelium functions as a physical barrier for the exchange of macromolecules, fluid, electrolytes and cells between surrounding tissue and the inner vascular space. Through the expression of various secreted factors and junctional or adhesion molecules, it also regulates leukocyte adhesion, trans- endothelial migration and smooth muscle function. The vascular endothelium is responsive to various stimuli and therefore consists of a dynamically mutable interface. The endothelium is affected by blood components and flow (Evans and Kwak, 2013).

The stimuli can have either biochemical origin, such as proinflammatory cytokines and oxidized lipoproteins or can be generated by flowing blood, which varies according to anatomical location (Gimbrone, 1995, Gimbrone et al., 1999).

Two main hypotheses may explain the observation of lesion development in areas with altered blood flow. The first theory, "the mass transport theory" or "the response to influx theory" describes an association between increased time of exposure and increased rate of bioactive substances uptake. Low or disturbed flow may lead to an increase of the time that endothelial cells are in contact with the blood and its bioactive components, such as LDL and nitric oxide. As a result, the cells have more time to respond to or "absorb" these potential atheromatous materials causing atherogenesis (Back, 1975). Animal experiments have shown a greater influx of tagged macromolecules in areas prone to atherosclerosis, such as low blood flow areas, thereby supporting the mass transport theory (Tarbell, 2003).

The second hypothesis is called "the shear stress theory". Different types of haemodynamic forces, such as hydrostatic pressures, cyclic strains and wall shear stresses (WSS) can be generated by pulsatile blood flow through the arterial vasculature (Topper and Gimbrone Jr, 1999).

Shear stress (τ) is measured in N/m^2 Pascal (Pa) and arises from the friction between two virtual layers in a fluid and is promoted by the difference in movement of the two layers and the roughness between these layers. The *in vivo* estimation of wall shear stress, without complex calculations, is based on the Hagen- Poiseuille formula: $\tau = 4\mu Q / \pi R^3$, where τ is shear stress, μ the dynamic blood viscosity, Q the volume flow- rate and R the inner radius of the conduit cylindrical tube.

As the blood, a viscous fluid, comes in contact with the cellular layer, frictional forces (wall shear stresses) are exerted on the endothelium. Shear stress depends on the direction, time and the magnitude of the pulsatile nature of blood flow and vascular anatomy. Different blood flow patterns lead to both spatial and temporal alterations in wall shear stress profile. To be more specific, uniform laminar flow of high shear stress occurs in the unbranched areas of medium sized arteries, whereas disturbed complex flow of low shear stress occurs at branch points and major curvature of the arterial tree (Figure 1.1) (Davies, 2009, Chiu and Chien, 2011).

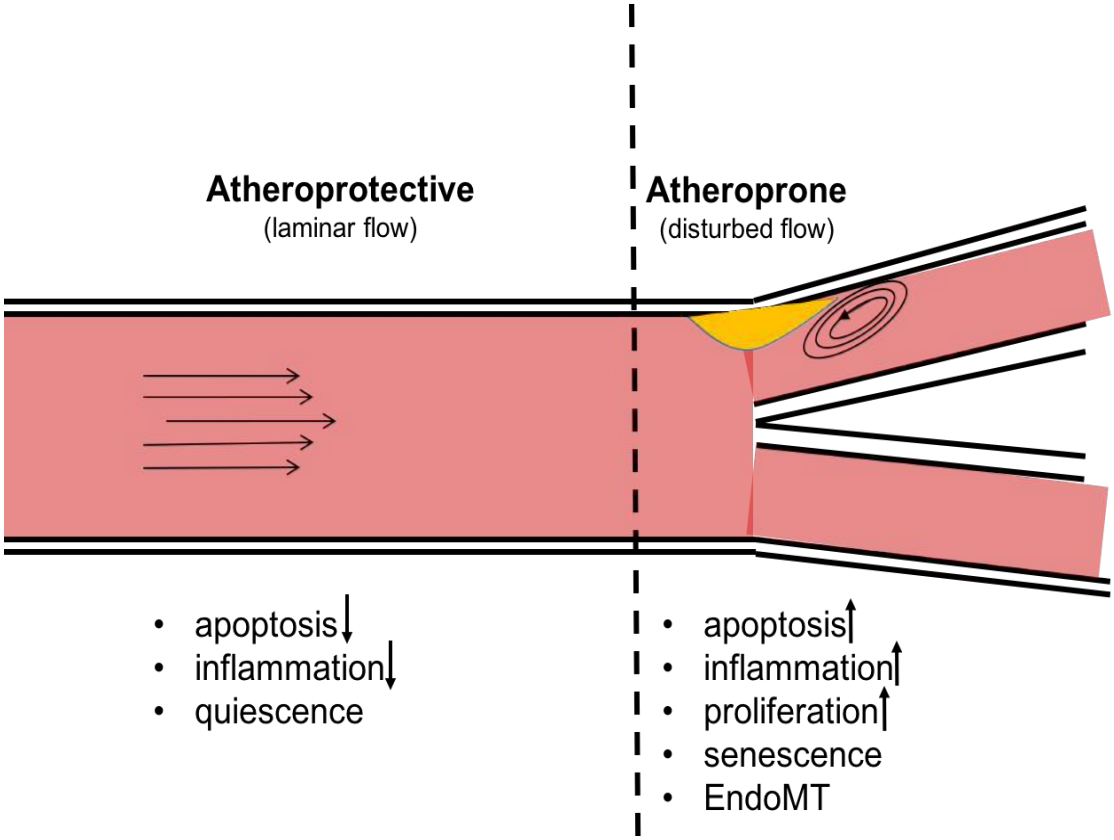


Figure 1.1 Schematic representation of the link between flow dynamics and atherosclerosis

Unidirectional wall shear stress (laminar flow) occurs in unbranched areas of arteries, which are protected from atherosclerosis, whereas oscillatory shear (disturbed flow) is found in branched points, which are thought to be atheroprone.

However, the hypothesis proposing the possible relationship between flow, geometry of vasculature and atherogenesis was and remains a controversial issue. In 1969, Fry suggested that high wall shear stress can damage the arterial endothelium and can lead to pathology (Fry, 1969). At that time, Caro proposed that lesions are formed in areas of disturbed blood flow and high WSS is protective (Caro et al., 1969). This theory concludes that oscillatory flow and low shear stress is a risk factor for developing atherosclerosis while laminar flow and high shear stress is atheroprotective, highlighting the important role of both magnitude and flow pattern into atherogenic process. Although oscillatory flow and low shear stress often overlap spatially in arteries, the contribution of each pattern to the initiation of atherosclerosis remains unclear. To be more specific, a systematic review of papers that compare the spatial distribution of atherosclerotic plaques and the local hemodynamic forces was unable to distinguish whether flow direction (oscillatory flow) or shear stress magnitude (low shear) is the key factor contributing to atherogenesis (Peiffer et al., 2013b). Both these factors (low WSS and oscillatory flow) are uniaxial and do not take into account the multidirectional nature of “disturbed flow”. In order to better predict the areas of developing plaques in vasculature, transverse wall shear stress (transWSS) has been used to quantify the multidirectional character of “disturbed flow” (Peiffer et al., 2013a). TransWSS is defined as the average magnitude of WSS components acting transversely to the mean vector. Studies have shown that transWSS can be used as a strong predictor of atherosclerotic lesion prevalence, whereas oscillatory shear index (an index to characterize uniaxial oscillatory flow) or time- average wall shear stress (TAWSS) had no correlation with lesion prevalence (Mohamied et al., 2015). One of the key findings of this study is that oscillatory flow applied perpendicularly to the long axis of the endothelial cells has a pro- inflammatory effect, whereas application of oscillatory flow in parallel to the endothelial axis has an anti- inflammatory effect.

Therefore, both the direction and magnitude of flow contribute to the initiation of the disease but further studies are required in order to distinguish, which specific hemodynamic factor affects atherogenic pathways leading to increased inflammation, apoptosis etc. (Figure 1.1).

Several studies have shown that the expression of various genes, particularly related to inflammation, is affected by shear stress (Hajra et al., 2000, Passerini et al., 2004, Partridge et al., 2007). Proinfammatory events through the action of cytokines such as tumor necrosis factor- alpha (TNF-α) occur during atherogenesis (Sheikh et al., 2003). Nuclear factor kappa-B (NF- κB) and mitogen- activated protein kinase (MAPK) pathways are two inflammatory pathways regulated differentially according to the local blood flow pattern (Chiu and Chien, 2011). Inflammation and subsequently atherogenesis can be suppressed by high shear stress through regulation of MAPK and NF- κB signaling pathways.

Shear stress can also induce molecular pathways associated with apoptosis or senescence. Apoptosis can be induced by disturbed flow through several mechanisms some of which include the activation of JNK MAP kinase, PKCζ and p53 (Magid and Davies, 2005, Garin et al., 2007, Chaudhury et al., 2010, Heo et al., 2011). On the contrary, apoptosis is suppressed through the activation of known protective signaling pathways, such as pathways including the NO synthase and superoxide dismutase (Liu et al., 2001, Jin et al., 2002). Although a study of Warboys showed that EC senescence is induced by low oscillatory shear stress via a p53 related pathway, other studies have shown higher EC proliferation rate in atheroprone sites (Foteinos et al., 2008, Warboys et al., 2014, Schober et al., 2014). These findings reveal the complexity and heterogeneity of ECs at atheroprone sites.

Endothelial to mesenchymal transition (EndoMT) is another biological process regulated by shear stress affecting the progression of atherosclerosis. To be more specific, a recent study by Mahmoud has found that TWIST1, a gene involved in EndoMT, was expressed preferentially at disturbed flow regions and promoted atherosclerosis by enhancing inflammation (Mahmoud et al., 2016). Another study showed that dysregulation of the endothelial FGFR signaling pathway by oscillatory flow promotes EndoMT contributing to the progression of atherosclerosis (Chen et al., 2015).

In summary, although there is evidence to support the mass transport theory, recent studies have highlighted the importance of shear stress in endothelial cells pathophysiology. Therefore, a crucial question is how endothelial cells can sense different blood flow patterns.

1.3 Mechanoreceptors on endothelial cells

Endothelial cells can sense changes in shear stress and consequently change their function and morphology (Ando and Yamamoto, 2009). The shear stress changes are transmitted by specific mechanoreceptors into the inner cell and activate downstream signaling pathways (Davies and Tripathi, 1993, Liu et al., 2002, Zhou et al., 2013). Although some of the signaling pathways activated by shear stress are well described, the sensors and mechanisms by which ECs detect shear stress are incompletely understood.

There is a variety of candidates for shear stress sensors. Candidate mechanoreceptors can be either molecules expressed on the cell membrane including adhesion molecules, ion channels, surface receptors, caveolae, primary cilia and the glycocalyx or molecules supporting cell structures such as the lipid bilayer itself and the cytoskeleton (Figure 1.2). Known molecules that are expressed on endothelial cell surface, their response to force (mechanical, magnetic etc.) has been proved and their mechanostimulation causes subcellular events, such as Ca^{2+} influx and upregulation of certain molecules, for example NF- κB, are listed in Table 1.1. as known endothelial mechanoreceptors.

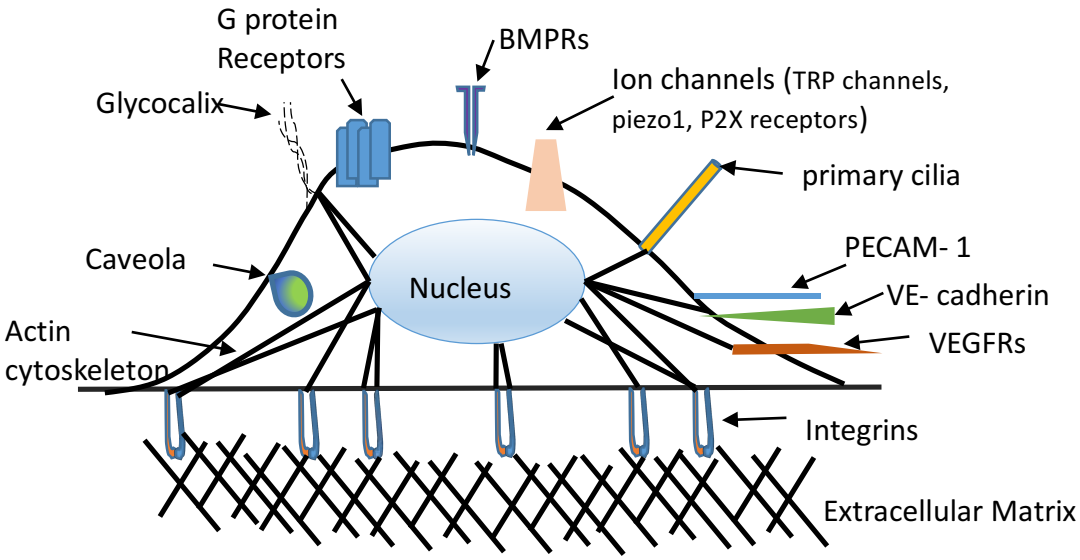


Figure 1.2 Schematic representation of known endothelial mechanoreceptors

Mechanoreceptors can be found on both the apical and the basal membrane. They can also be at the cell junctions or intracellularly.

Table 1.1 Known mechanoreceptors expressed in endothelial cells

Type of mechanosensor	Name of mechanosensor	Shown to sense force directly (technique)	Relative literature
Ion channels	TRPV4 Piezo1 P2X4 (indirect activation) $I_{K.S}$	YES (patch clamp) YES (magnetic beads) NO YES (patch clamp)	(Olesen et al., 1988, Yamamoto et al., 2003) (Mergler et al., 2011) (Li et al., 2014)
G protein receptors	Angiopoietin (Tie-2) VEGFR2	NO NO	(Shay-Salit et al., 2002) (Lee and Koh, 2003)
Cell adhesion molecules	β_{1-} integrin $\alpha_v\beta_3$ integrin PECAM-1 (complex with VEGFR2 and VE- cadherin)	YES (magnetic beads) NO YES (magnetic beads)	(Tzima et al., 2001) (Gloe et al., 2002) (Tzima et al., 2005, Collins et al., 2012, Collins et al., 2014)
Primary cilia	PKD2/PC2/TRPP2	YES (high-resolution imaging of live zebrafish embryos)	(Goetz et al., 2014)
Caveolae	Caveolin-1	NO	(Spisni et al., 2001) (Yu et al., 2006) (Radel and Rizzo, 2005)

Glycocalyx	Heparan sulfate proteoglycan Syndecan 4	NO	(Florian et al., 2003) (Baeyens et al., 2014)
BMPRs	BMPRII BMPRIB	NO NO	(Zhou et al., 2012) (Zhou et al., 2013)
Cytoskeleton	Actin, MTOC	YES (atomic force microscopy)	(Barbee et al., 1994, Mathur et al., 2000) (Goehring and Grill, 2013)
Nucleus	LINC complexes	NO	(Tkachenko et al., 2013) (Chambliss et al., 2013)

1.3.1 Ion channels

A variety of ion channels, expressed on ECs plasma membrane, can be activated by shear stress and as result can be characterized as potential mechanoreceptors. Nakache used a voltage- sensitive dye to confirm EC membrane hyperpolarization in response to shear stress (Nakache and Gaub, 1988). The hyperpolarization of the cell membrane can cause the influx of extracellular Ca^{2+} into the cell. Ca^{2+} is an important messenger molecule that mediates the activation of multiple downstream molecular pathways (Schwartz, 1993, Hoyer et al., 1996). In mammals, many transient receptor potential (TRP) ion channel family members have been reported to act as mechanoreceptors and be involved in mechanotransduction (Yin and Kuebler, 2010). TRPP1/TRPP2, TRPV4, TRPC3 and TRPM7 have been found to be localized on endothelial cells and to respond to shear stress (Oancea et al., 2006, Liu et al., 2006, Köhler

et al., 2006, Nauli et al., 2008). A study of Li revealed that Piezo1 channels can sense shear stress and as a consequence calcium enters into ECs and mediates other molecular events, such as the activation of calpain-2 that can lead to cell shape rearrangement and cell alignment (Li et al., 2014). A recent study of Matthews has reported that TRPV4 ion channels interact and “cooperate” with cell surface β1- integrin (Matthews et al., 2010). This group found a Ca^{2+} influx via activated TRPV4 ion channels in response to mechanical force applied to integrins.

This example shows that several mechanosensor candidates can function not only individually but also cooperatively. Interestingly, ion channels can be activated by shear stress indirectly. An example of this indirect activation is the P2X4 receptors expressed on the EC plasma membrane. P2X4, a subtype of ATP- cation channels, are activated by ATP released from the ECs after their exposure to shear stress. The ATP-dependent activation of the P2X4 receptors opens the P2X4 ion channels and allows to Ca^{2+} to enter into ECs (Yamamoto et al., 2000, Yamamoto et al., 2003, Wang et al., 2015).

1.3.2 G protein receptors

Many cell G proteins receptors can act as mechanotransducers. For instance, VEGFR2, a receptor- tyrosine kinase (RTK), is expressed on EC plasma membrane and is activated by rapid phosphorylation after application of flow (Shay-Salit et al., 2002). The activation of VEGFR2 in response to flow induces the formation of membranal clusters and its binding to the adaptor protein Shc (Chen et al., 1999). In addition, shear stress mechanotransduction can involve GTP binding protein- coupled receptors (GPCRs) (Gudi et al., 1996, Jo et al., 1997). Experiments on bovine aortic ECs showed that GPCRs change their configuration in response to shear stress. However, it is not clear if there is a direct effect and interaction of shear stress on G proteins.

1.3.3 Caveolae

Vascular endothelial cell, amongst other cell types, have caveolae at their plasma membrane surfaces. Caveolae are small (50- 100 nm) flask-shaped invaginated structures. They are rich in caveolins, sphingolipids and cholesterol and they are thought to play an important role in intracellular transport of substances (Shaul and Anderson, 1998). *In vitro* studies with cultured cells exposed to flow has demonstrated a potential role of caveolae in mechanotransduction (Sun et al., 2002). The exact role of caveolae is still unknown but their "activation" by shear stress may induce other signaling molecules, such as ERK and eNOS, or enhance Ca^{2+} signaling in an ATP dependent mechanism in ECs (BURNSTOCK, 1999, Boyd et al., 2003, Yamamoto et al., 2007).

1.3.4 Primary cilia

Cilia protrude from the cell surface and are membrane- covered rod-like organelles. There are two main types of cilia: the motile and non- motile cilia, also known as primary cilia that can function as sensory organelles. Their core consists of nine doublet microtubules, which are connected with cytoplasmic microtubules (Ishikawa et al., 2012). Hierck found that the expression levels of the transcriptional factor Kruppel- like factor-2 (KLF-2), which is thought to be atheroprotective, are increased in ECs that have primary cilia exposed to shear stress, whereas the upregulation of KLF-2 failed to occur in ECs that did not have primary cilia (Hierck et al., 2008). In addition, a study from Iomini showed that primary cilia were disassembled by high laminar shear stress (Iomini et al., 2004).

The bending of cilia caused by shear stress can either result in cytoskeletal rearrangements or activate the opening of local channels leading to Ca^{2+} influx (Nauli et al., 2008, AbouAlaiwi et al., 2009).

A recent study of Goetz in zebrafish found that mechanotransduction involves the calcium- permeable channel PKD2/PC2/TRPP2, which is a component of primary cilia (Goetz et al., 2014). Moreover, *in vivo* studies with apolipoprotein- E- deficient mice showed that endothelial primary cilia were not present in the areas of high shear and uniform flow, which is thought to be atheroprotective, whereas the number of endothelial primary cilia was enriched in areas with low shear and disturbed flow, which are considered to be atheroprone (Van der Heiden et al., 2008). However, the physiological significance of mechanotransduction via primary cilia remains uncertain.

1.3.5 Glycocalyx

The glycocalyx contains glycosaminoglycans (GAGs) and is a highly charged extension of the cell surface, which is attached to the cytoskeleton (Weinbaum et al., 2007). Key components of the glycocalyx structure may participate in mechanotranduction. The removal of the glycosaminoglycan heparin sulfate and hyaluronic acid glycosaminoglycans, all components of the glycocalyx, dysregulate the flow dependent endothelial NO production and may affect downstream molecular pathways (Florian et al., 2003, Yao et al., 2007, Pahakis et al., 2007). Furthermore, the glycocalyx spatial distribution on the cell surface may be associated with the local shear stress pattern (Yao et al., 2007). A study from Koo showed that high uniform flow induced the expression of the endothelial glycocalyx on the endothelial apical surface (Koo et al., 2013). On the other hand, the application of low oscillatory flow caused a decrease in the expression of the glycocalyx. Moreover, a study from Baeyens demonstrated that knock down of syndecan- 4, which is an important component of the glycocalyx, led to activation of pro- inflammatory responses in endothelial cells, resulting in the development of atherosclerotic lesions in normally atheroresistant areas (Baeyens et al., 2014).

Thus the glycocalyx is a good candidate for sensing distinct mechanical force, but further studies are essential to confirm its role in mechanotransduction.

1.3.6 BMPRs

Bone morphogenic protein receptors (BMPRs) are a family of transmembrane serine/ threonine kinases, essential for Smad signalling (Miyazono et al., 2005). Combining *in vitro* and *in vivo* experiments, studies have shown that BMPRs act as mechanosensors, by activating the BMPR-specific Smad1/5 signalling pathways (Zhou et al., 2012). To be more specific, exposure of endothelial cells to oscillatory flow led to BMPRII activation, which induced the association between BMPR1B and $a_v\beta_3$ integrins. As a result, smad1/5 is phosphorylated and mTOR is activated leading to increased proliferation (Zhou et al., 2013). As increased proliferation is a characteristic of regions prone to the development atherosclerosis, BMPR- specific Smad1/5 activation by oscillatory flow, can be a hemodynamic- based therapeutic target.

1.3.7 Plasma lipid membrane

The plasma lipid bilayer membrane is in a liquid-crystalline-like state and its properties can alter continuously due to several factors, such as temperature, pH, content of water, membrane cholesterol and changes in lipid composition and packing (Lenaz, 1987, Phillips et al., 2009). It is possible that the regulation of molecules connected directly or indirectly to the plasma membrane may be affected by the properties of the membrane. As a result, if the membrane properties change in response to shear stress, crucial molecules may be activated and change cellular behavior (Haidekker et al., 2001).

1.3.8 Endothelial cytoskeleton

The cells can exert stresses, generated in the contractile cytoskeleton, to the extracellular matrix (ECM) (Ingber, 1997, Geiger et al., 2001). Stresses can also be transmitted from the ECM to the cells. As a result, the shape of the cells and structure of the cytoskeleton can be influenced by mechanical forces.

It is proposed that cells may use a special building system, which was first described as an architectural model by the architect Buckminster Fuller (Ingber, 1993). This “intracellular” construction depends on tensional integrity (tensegrity). This model is based on the continuous interaction of rigid struts, which are connected by consecutive series of elastic tensile threads. The tensegrity model can predict changes of cell shape and explain structural patterns observed in the cytoskeleton of living cells.

As the forces on the cell surface increase, the mechanically-independent structural elements of the tensegrity model rearrange without loss of tensional consecutiveness or topological disorganization. The endothelial cytoskeleton as a whole is an interesting candidate and may function as a “general mechanoreceptor” or “mechanoreceptor signal transducer” because it is connected with several single mechanoreceptors (Ali and Schumacker, 2002). The cytoskeleton may be a universal structure connecting all shear stress sensors together and transmit mechanical force to specific inner subcellular compartments (Helmke and Davies, 2002). There is a constant tension across the entire endothelial cytoskeleton due to the complex network of intermediate, microtubules and actin fibres in the cell. This tension can be redistributed by shear stress and the members of this network may be rearranged in a way that does not change the cell shape and behavior (Ingber, 1997). However, the redistribution of the force can be translated into downstream chemical signals.

1.3.9 Cell adhesion molecules

ECs can sense shear via multiple mechanosensors, one of which is found at cell- cell junctions and consists of PECAM-1, VE-cadherin and VEGFR receptors (Tzima et al., 2005, Conway et al., 2013). PECAM-1 senses and converts the mechanical force into biochemical signals via activation of a src family kinase, which phosphorylates and activates VEGFRs (independent of ligand), leading to downstream signals. Interestingly, these signals include activation of both protective eNOS and inflammatory NF-kB. VEcad functions as an adaptor that is required for VEGFRs activation via an interaction between their transmembrane domains (Coon et al., 2015).

Numerous *in vivo* studies support the role for these proteins in flow-dependent vascular remodeling and atherosclerosis (Goel et al., 2008, Harry et al., 2008, Chen and Tzima, 2009, Chen et al., 2010). There are also hints that this complex plays a key role in sensing flow direction. First, a polarity complex, consisted of Par3/Par6/Tiam1/p67phox interacts with the mechanosensory complex via binding to VEcad, and is involved in flow activation of NF-kB (Liu et al., 2013). These proteins localized to the downstream edge of the cells relative to the direction of flow and their assembly was dependent on the direction of flow. The junctional complex also determines EC sensitivity to flow magnitude, as the level of the VEGF receptors determines the fluid shear stress “set point” at which flow stabilizes the vasculature (Baeyens et al., 2015). For example, human umbilical vein endothelial cells align efficiently around 10 dynes/cm^2, whereas human dermal lymphatic endothelial cells, which express higher levels of VEGFR3, align effectively at 5 dynes/cm^2. Therefore, the expression levels of VEFGR3 determine the flow sensitivity of distinct endothelial cell types.

In summary, candidate mechanoreceptors can include many different molecules either on the surface of cells or within the inner cell. Although, there is some evidence indicating the function of some molecules as mechanoreceptors, few studies have identified the exact mechanism for their direct activation by force and downstream effects are still unknown. Indeed, it is crucial to clarify how these mechanoreceptrors sense and transmit different patterns of flow and how they may change their structure in response to different forces.

1.4 The integrins

The integrins consist a superfamily of cell adhesion receptors. They are transmembrane αβ heterodimers that can recognise cell-surface ligands and extracellular matrix ligands (Hynes, 2002). The RGD sequence included in fibronectin of the extracellular matrix is the main integrin-binding motif (Pierschbacher and Ruoslahti, 1984). In addition, integrins can also bind to non-RGD sequences, such as the LDV peptide of the vascular adhesion molecule 1 (VCAM-1), which is often expressed in inflammatory regions of the endothelium (Elices et al., 1990). There are 18 α and 8 β subunits in the human genome (Takada et al., 2007). There is no detectable homology between subunits α and β. Both α and β gene families have been developed by gene duplication, with a sequence identity of 30% among α subunit and 45% among β subunit (Huhtala et al., 2005).

The crystal structure of $\alpha_{II}\beta_3$ and $\alpha_V\beta_3$ human integrins revealed multiple domains in the extracellular part of the integrin heterodimer (Xiong et al., 2001, Xiong et al., 2002, Xiao et al., 2004). The ligand- binding site is located in the headpiece of $\alpha_V\beta_3$ and contains the α_V subunit, which is consisted of a β- propeller domain and a plexin- semaphorin- integrin (PSI) domain and the β subunit including the βA and the hybrid domain.

The cytoplasmic tail of integrins is less than 75 amino acids long with an exception of β4 integrin (approximately 1,000 amino acids). The original crystal structure of $\alpha_V\beta_3$ integrin showed that the head region takes a bent conformation in the low-affinity for the ligand state (inactive form, Figure 1.3 A). It is proposed that active integrins take an extended form that enables the binding of the ligand and the transmission of the signal (Figure 1.3 B) (Campbell and Humphries, 2011).

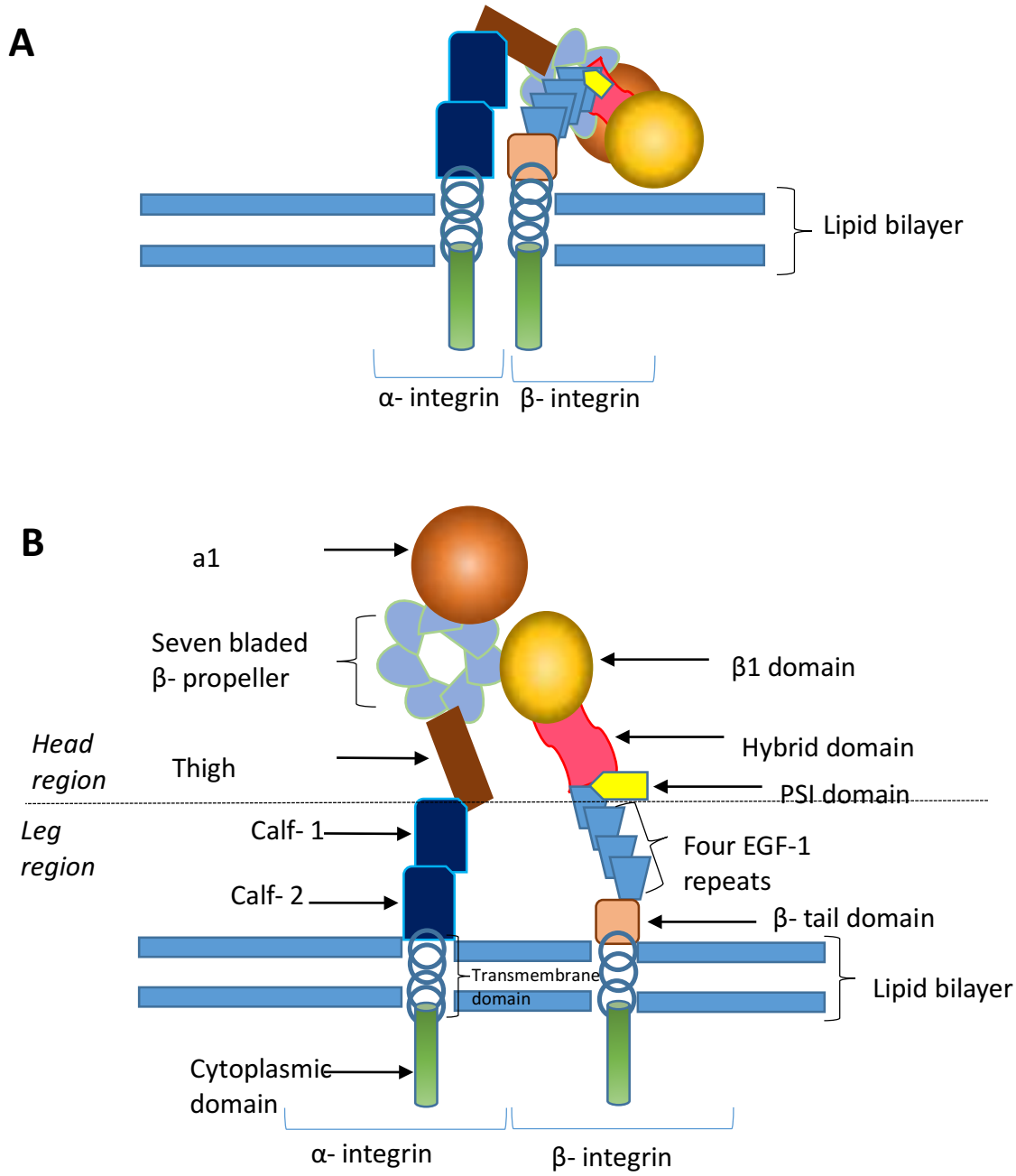


Figure 1.3 Schematic representation of a_vb_3 structure and its activation status

In its inactive form the molecule takes a closed conformation (A) but after its activation the heterodimer takes an extended conformation (B). The crystal structure of $\alpha_V\beta_3$ human integrins revealed multiple domains in the extracellular part of the integrin heterodimer shown in (B).

Integrins can generate intracellular signals with the binding of extracellular ligands (outside- in signaling). When a ligand, such as the RGD sequence of fibronectin binds to integrins extracellularly, several proteins such as talin, kindlin and vinculin, are recruited to integrin tails intracellularly. Talin and kindlins, two families of the FERM- domain proteins, are recruited to the cytoplasmic tails of integrins and then bind to the N- terminus of vinculin, which binds to actin filaments creating a connection between integrins and the cytoskeleton (Moser et al., 2008, Calderwood et al., 2013). This connection is crucial for the communication of the extracellular environment and the inner cell (Wegener et al., 2007). The ligand affinity of integrins is depended on both the heterodimeric structure and cytoplasmic signals. The binding of extracellular ligand itself can cause the clustering of integrins on the cell membrane, an event leading to intracellular signals. As a result, the cytoplasmic tails interact with intracellular signaling molecules, such as enzymes (Ras and Rho) and adaptors (paxillin and Cas/Crk) binding cells to the ECM (Arnaout et al., 2005, Shattil, 2005) Therefore, the contact sites between the ligand and the integrin are active sites transducing information about the extracellular ligand density or the direction and magnitude of extracellular forces exerted on the cell surface. However, there is no evidence showing a ligand-independent activation of integrins, triggered by extracellular mechanical forces.

Moreover, integrins can be regulated by signals coming from the inner cell (inside- out signaling). The intracellular activation of integrins is caused by the phosphorylation of the cytoplasmalic domain of the β subunit. This phosphorylation event is triggered by signals from G- protein coupled receptors and leads to disassociation of the α and the β cytoplasmic tails that normally maintain the integrin structure in the inactive state (Humphries, 1996, Ginsberg et al., 2005). This disassociation of the cytoplasmic tails induces a structural change in the extracellular part of the integrin and subsequently the affinity for its ligand is increased.

1.4.1 Integrins and shear stress

As far as the endothelial cells and the responses to fluid shear stress is concerned, integrins seem to play a crucial in mechanotransduction and may act as mechanoreceptors. Endothelial cells maintain their stable position and resistance to blood flow because they are anchored to the subendothelial ECM. One proposed model for the role of integrins in shear stress signaling is that the generated tension from the apical surface is transmitted through the cytoskeleton to the integrins based on the basal surface. Subsequently, integrins change their conformation and transduce downstream signals (Davies, 1995).

Several molecular pathways, which are regulated by the binding of integrins to ECM, can also be stimulated by shear stress. Shear stress has been found to activate β1- integrin triggering endothelial cell changes, such as cytoskeletal alignment in a Rho dependent and MAP kinase manner (Ishida et al., 1996, Tzima et al., 2001). Integrins also mediate the activation of the small G- protein RhoA via a shear stress induced mechanism. The activation of RhoA results in the activation of the sterol regulatory element- binding protein, a transcription factor regulating multiple genes related to fatty acid and cholesterol biosynthesis (Liu et al., 2002). Furthermore, shear stress stimulates the activation of c-Src, FAK and the tyrosine phosphorylation of $p130^{cas}$, all of which are also involved in the integrin signalling pathway. Integrins can also interact with other mechanoreceptors, such as VEGFR- 2 which is part of the mechanosensory complex (Tzima et al., 2005). A study from Wang showed the role of β_1 and $\alpha_v\beta_3$ integrins in VEGFR- 2 activation in ECs under shear stress (Wang et al., 2002). Moreover, the shear stress induced activation of β1- integrin causes the tyrosine phosphorylation of the caveolin-1, a caveolar structural protein.

Finally, experiments in a study from Matthews included the direct application of force of different magnitudes (100- 2000 pN) on RGD- coated magnetic beads bound on integrins heterodimers (Matthews et al., 2010). The application of force induced a rapid calcium response localized around the bead area. This result supports the idea that mechanostimulation of integrins trigger subcellular events.

In conclusion, there is evidence suggesting that integrins play an important role in mechanotransduction of shear stress induced signals. However, the detailed mechanisms of how mechanical forces of different directions are sensed, transmitted and eventually transformed into chemical signals remain unknown. Therefore, intensive study of integrin dynamics is essential to enlighten current questions.

1.5 Study of vascular mechanobiology in three dimensions

A challenge in the study of molecular signaling pathways and cell function in response to force/ flow has been the development of techniques to control and manipulate specific cellular structures, which requires input from biologists, engineers and physicists. Various tools have been developed to explore the cellular behavior under mechanical stimuli. Different groups have used different techniques, such as atomic force microscopy, patch clamp trapping, fluid jets, optical micromanipulation and magnetic tweezers (Desai et al., 2007).

Biomechanics of living cells can be studied with the use of atomic force microscopy (Mathur et al., 2001, Radmacher, 2002). The atomic force microscopy is a cantilever- based scanning probe that can have two initial modes. The displacement mode gives the ability to the operator to perform mechanical tests on cells and especially local pulling following adhesion of the probe. The constant force mode allows the mapping of the cell surface topography (Mathur et al., 2000).

The use of patch clamp technology has enabled the observation of single ionic channels (Neher and Sakmann, 1976). In this technique, a small heat- polished glass pipette is used to apply a pressure on a very small area of the cell membrane and consequently an electrical seal is formed (Zhao et al., 2008). A small patch of membrane is formed into the pipette due to high resistance (approximately 50 MΩ) of the seal. The use of a special cell buffer solution and through the electrodes in the micropipette, voltage specific measurements can be taken (voltage- clamp configuration). These measurements can give information about the ion influx into or out of the cell. In 1988, Olesen having used the patch clamp technique, identified a shear- stress activated K^+ ion channel which functions as a mechanosensor in arterial endothelial cells(Olesen et al., 1988).

The fluid jet technique includes the use of a single micropipette, which is positioned above the cell, very close to the cell surface, so that a jet of fluid can be applied over the membrane. The distance between cell surface and the tip of micropipette, the jet centerline velocity and the internal micropipette diameters dictate the force that is applied. Cells are held on a micropipette and an ejecting pipette applies the fluid jet on the cell surface. Changes on the surface after the ejection of the fluid jet, can be measured to give information about cell surface mechanosensing characteristics (Moazzam et al., 1997).

The micromanipulation of microscopic objects and biological molecules can be controlled with the use of optical forces. Ashkin was the first who used optical traps for biomanipulation and specifically the manipulation of viruses and bacteria (Ashkin and Dziedzic, 1987). In this technique, a force is generated in a single- beam optical trap by a laser and can act on small particles (Steubing et al., 1990). However, this technique has also been used for the study of cells, for example by trapping integrin-bound beads on the surface of cells (Choquet et al., 1997, Desai et al., 2007).

In summary, there are several methods to study the mechanical properties of living cells and to detect the mechanical sensors that they possess including AFM, patch clamping, fluid jets and optical tweezers. Magnetic based techniques are also important and are at focus of my studies; they are discussed in the next section.

1.6 Use of magnetic nanoparticles and study of force applied on cells

The development of magnetic micro- and nanoparticles has enabled the mechanical properties of the cell to be probed by magnetically generated stresses (Figure 1.4) (Lele et al., 2007).

Magnetic nanoparticles have custom sizes, ranging from a few up to tens of nanometers. There are several advantages using magnetic nanoparticles for studying mechanical forces applied onto a cell. Their size means that they can be applied close to the biological structure of interest, such as single cells. Magnetic nanoparticles can be coated with unique molecules and as a result they can target, be attached and interact with certain molecular structures of the cells. They can also be controlled by an external magnetic field gradient due to their magnetic properties. Furthermore, the range and time of the magnetic field application can vary, being externally controlled, and consequently cell responses to different mechanical forces can be examined (Pankhurst et al., 2003).

The use of magnetic nanoparticles to study mechanical forces applied to cells date back to 1922 and 1924 with the studies of Heilbronn and Seifriz respectively (Heilbronn, 1922, SEIFRIZ, 1924). Kirschvink suggested a theoretical mechanism for activating mechanosensitive ion channels in the human brain with the use of biogenic magnetite nanoparticles stimulated by weak magnetic fields (Kirschvink et al., 1992).

In addition, another approach is “magnetic twisting cytometry”, which refers to the application of magnetizing pulse to the magnetic nanoparticle and results to its consecutive magnetization (Wang et al., 1993, Wang and Ingber, 1995).

The application of a “twisting field” gives the ability to twist the nanoparticle, which is attached to a receptor of interest on the plasma membrane, and investigate the mechanical cellular response. Other groups used “magnetic twisting cytometry” to activate mechanosensitve ion channels (Pommerenke et al., 1996, Lo et al., 1998, Hughes et al., 2005).

Their approach included the application of controlled forces to change the conformation, from open to close, of various ion channels. A more recent study of Hughes tried to examine the cellular response by targeting and stimulating specific mechanosensitive ion channels (Hughes et al., 2008). Moreover, Matthews developed a new technique, which contains the use of a magnetic needle to apply focused magnetic force on a very small area of the cell surface (Matthews et al., 2004). Applying this technique, they succeeded to promote membrane clustering of specific plasma membrane receptors (Mannix et al., 2008). Furthermore, Tzima used magnetic beads coated with antibodies to test whether specific receptors on endothelial cells surface can directly transduce force (Tzima et al., 2005). In a larger scale, a magnetically controlled cellular microchip was built by Ingber’s group and used to boost adhesion of cells coated with magnetic nanoparticles (Polte et al., 2007). This magnetic array can be applied to investigate simultaneously the behavior of multiple cells under the mechanical activation of various cell-attached ligands.

In summary, the study of the effects of force on cells requires the development of innovative techniques that can mimic physiological conditions. A variety of methods has been proposed but it is necessary to either improve current techniques or develop new “magnetic platforms” that will allow the deeper and more sufficient study of how mechanical forces are sensed and transmitted on the cells.

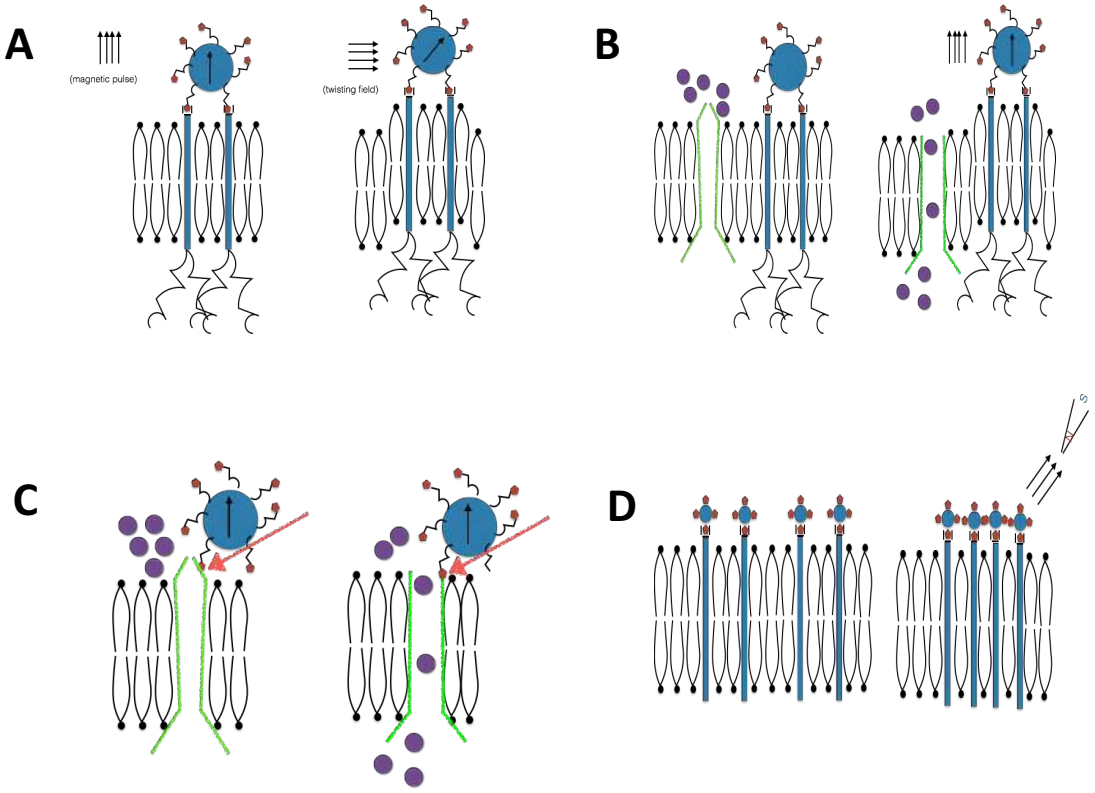


Figure 1.4 Schematic representation of current methods using magnetic nanoparticles to study mechanical forces applied to cells

A) Twisting cytometry: application of magnetizing pulse to a specific magnetic nanoparticle bound to a specific receptor results to the stretching of cellular membrane. B) Use of twisting cytometry to activate a mechanosensitive ion channel. C) Targeted activation of a mechanosensitive ion channel via application of magnetic field to a magnetic nanoparticle attached to the ion channel. The binding of the magnetic nanoparticle to the ion channel causes the opening of the channel and the influx of various molecules (red arrow). D) Clustering of receptors bound to magnetic nanoparticles. The application of magnetic field via a magnetic needle on the nanoparticles causes the clustering of the receptors.

1.7 Conclusion

In conclusion, the forces exerted on parts of the cardiovascular system have significant effects on the cardiovascular physiology and disease. Several haemodynamic factors and fluid patterns are linked to the initiation and progression of atherosclerosis. Although various studies found an association between oscillatory and/or low shear stress and atherosclerosis, further studies are required to clarify specific characteristics and factors, such as direction and magnitude, which may affect the process of atherosclerosis. An additional challenge is the discovery of new mechanoreceptors. The identification of their molecular function and structure will reveal new insights of the cellular mechanisms contributing to cardiovascular physiology and disease. A limiting factor of these studies could be the existing techniques for direct force application to vascular cells. Therefore, it is required the development of new methods that will give the ability to the operator to control the magnitude, direction and the amplitude of the mechanical force.

A crucial question that my work will try to address is the possible mechanism that endothelial cells of the vasculature use to distinguish different blood flow patterns and different types of stresses. The aim of my project is the characterization of mechanoreceptor(s) that respond to specific flow patterns in endothelial cells. This project will attempt to elucidate whether a single mechanoreceptor can sense different blood flow patterns or different receptors are required for each specific condition.

The discovery of both function and structure of a new mechanoreceptor on endothelial cell surface will give us the opportunity to gain deeper knowledge in the cardiovascular biology. Only if we understand the physiological mechanisms of vascular mechanotransduction, we will be able to interfere and may prevent pathological conditions, such as atherosclerosis.

Chapter 2: Materials and Methods

2.1 Reagents

Reagent	Vendor	Cat. number
14 gauge, 2x45mm cannula	Sentraflex	A/1101/14/P
15 µ- Slide I 0.4 Luer	Ibidi	80176
BD Precisionglide® syringe needles	Sigma-Aldrich	Z192503
34mm diameter 6- well plates	Costar®, Sigma-Aldrich	CLS3516
384 well plate	Anachem	95016339
4', 6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich	10236276001
4% Formaldehyde	VWR	9713.1
60- µDish 35mm, low	Ibidi	80136
75 cm^2 filter capped cell culture flask	Thermo scientific	156499
96- well plate	Sigma-Aldrich	CLS3300
Amphotericin B	PAA	BP2645-50
Anhydrous DMSO	Sigma-Aldrich	276855
Bioline Isolate II RNA mini kit	Bioline	BIO-52072
Bovine Serum Albumin	Sigma-Aldrich	A7906
Cal-520™	AAT Bioquest®	21130-AAT
CFX3854™ Real-time instrument	BioRad	1855485
Clear adhesive film for 384 well plate	Anachem	17012021
Collagenase from Clostridium histolyticum	Sigma-Aldrich	C8051
DMEM	Sigma-Aldrich	D2429
Dynabeads® Goat anti- Mouse IgG	Invitrogen	11033
Dynabeads® M-450 Tosylactivated	Invitrogen	14013
Dynabeads® sheep anti- rat IgG	Invitrogen	11035
Endothelial cell growth supplement	Merck Millipore	02-101
Ethanol	Sigma-Aldrich	652261
Ethylene glycol-bis (2-aminoethylether)-N,N,N',N'- TETRAACETIC ACID (EGTA)	Sigma-Aldrich	E3889
Ethylenediamine Tetraacetate Acid (EDTA)	Thermo-Fisher	BP2482-500
FBS	Life Technologies	10500

Fibronectin	Sigma-Aldrich	F1141
Filtropur™ filter	SARDTEDT	20003477
Gelatin from bovine skin	Sigma-Aldrich	G9391
Goat serum	Sigma-Aldrich	G6767
Heparin sodium salt from porcine intestinal mucosa	Sigma-Aldrich	H3149
iScipt™ cDNA synthesis kit	BioRad	1708890
KAPA SYBR FAST qPCR Kit Master Mix (2x) ABI Prism™	KAPA Biosystems	KK4601
L- Glutamine	Gibco	BE17-605E
Lobind™ eppendorph tube	Eppendorph	Z666505
M199	Sigma-Aldrich	M0650
NBCS	Life Technologies	16010-159
Parafilm	Peckiney packaging	PM992
Penicillin/ Streptomycin	Invitrogen	15140-122
Phosphate Buffered Saline (PBS)	Sigma-Aldrich	P4417
Plastic syringes	Plastipak™	10636531
Pluronic® F-127	Invitrogen	P3000MP
Poly-D-lysine hydrobromide	Sigma-Aldrich	P6407
Prolong® gold anti-fade reagent	Invitrogen	P36930
RLT- lysis buffer	Qiagen	79216
RNase® zap	Ambion	AM9780
RNeasy kit	Qiagen	74104
ROCK Inhibitor (Y-27632)	Sigma-Aldrich	SCM075
Thapsigargin	Life Technologies	T9033
TO-PRO-3 Iodide	Life Technologies	T3605
TritonX™	Sigma-Aldrich	T8787
TrpLe trypsin™	Thermo-Fisher	12604013
U 73122 (Phospholipase C inhibitor)	Sigma-Aldrich	U6756
β-mercaptoethanol	Sigma-Aldrich	M3701

2.2 Antibodies

Antibody	Vendor	Cat. number
12G10 (mouse anti-human)	gift from M. Humphries Lab (University of Manchester)	
4B4 (mouse anti- human)	gift from M. Humphries Lab (University of Manchester)	
8E3 (mouse anti- human)	gift from M. Humphries Lab (University of Manchester)	
9EG7 (rat anti- human)	gift from M. Humphries Lab (University of Manchester)	
AlexaFlour™ 488 (donkey anti- rat)	Life Technologies	A-21208
AlexaFlour™ 488 (goat anti- mouse)	Life Technologies	A-11001
AlexaFlour™ 488 anti-mouse CD31	Biolegend	102514
AlexaFlour™ 568 (donkey anti- rat)	abcam	ab175475
AlexaFlour™ 568 (goat anti- mouse)	Life Technologies	A-11004
AlexaFlour™ 647 (goat anti- rat)	abcam	ab150159
HUTS4 (mouse anti-human)	gift from M. Humphries Lab (University of Manchester)	
K20 (mouse anti- human)	gift from M. Humphries Lab (University of Manchester)	
mAb13 (rat anti- human)	gift from M. Humphries Lab (University of Manchester)	
Mouse IgG	ThermoFisher	10400C
P5D2 (mouse anti-human)	Sigma- Aldrich	MAB1959
TS2/16 (mouse anti-human)	gift from M. Humphries Lab (University of Manchester)	

2.3 Primary cell culture and maintenance

2.3.1 Isolation of Human Umbilical Vein Endothelial cells (HUVECs)

Human vein endothelial cells (HUVECs) were isolated from veins of umbilical cords donated from women who gave their ethical consent previously (ethical approval: Sheffield REC 10/1308/25). Briefly, with the use of a syringe inserted in a 2x45 mm cannula, 10- 15 ml of serum free M199 media (Table 2.2) was flushed through the vein in order to remove blood clots. Crocodile clips were used to immobilize the cannula- syringe at the one end and to seal the distal end of the cord. Then, collagenase (1 mg/ml) from *Clostridium histolyticum* (previously sterile filtered with 0.2 μm Filtropur filter) was infused into the vein and incubated at room temperature for 10-15 minutes. After removal of the clip at the distal end, the extracted HUVECs were flushed into a 50 ml tube and were centrifuged at 160 relative centrifugal force (RCF) in 21 ^{O}C for 5 minutes. The cell pellet was resuspended in complete M199 media and was seeded onto 1% (w/v) gelatinized (Sigma) T75 flasks. Cells were incubated at 37 ^{O}C and after 24 hours were washed with phosphate buffered saline (PBS, Sigma) and fresh complete M199 media (Table 2.2) was added to them. Cells were incubated at 37 ^{O}C in a 5% CO_2 incubator (Leec, Touch 190S). As soon as HUVEC (passage 0) were 80-90% confluent, they were passaged.

2.3.2 Endothelial cell culture

Laboratory consumables, such as laboratory pipettes and flasks, were cleaned with 70% ethanol (Sigma) before each cell treatment. All cell culture maintenance was carried out in a laminar flow hood (Heraeus-LaminAir, HBB1228), also cleaned with 70% ethanol before use. The cells were maintained in complete M199 (Table 2.2) media and it was changed every 2 days. Complete M199 media was warmed in a water bath (JB Aqua12) at 37 ^{O}C before each treatment.

Cells were washed with PBS (Sigma) and 1 ml of pre-warmed Trypsin (TrpLe^{TM}, ThermoFisher) was added causing their detachment after 1-2 minutes of incubation at 37 ^{O}C in a 5% incubator. Their detachment was confirmed using a light microscope. Complete M199 media was added to neutralize trypsin. Cells were centrifuged at 160 RCF for 5 minutes (ALC®, PK120R). The pellet was resuspended in complete M199 media and split equally in 1:3 ratio into a 1% (w/v) gelatinized (Sigma) 75 cm^2 filter capped cell culture flask (Thermo Scientific). Cells of passage 2-4 were used for all experiments.

2.4 *In vitro* systems for mechanical stimulation of endothelial cells

2.4.1 ibidi^{TM} flow system

An ibidi^{TM} flow system was used to test the effects of specific flow patterns on ECs. HUVECs isolated from umbilical cords were harvested from a confluent T75 flask and resuspended in 1000 µl complete M199 media (Table 2.2). The cell suspension was diluted in Trypan blue in a ratio of 1:10. A Neubauer haemocytometer was used to count the exact number of cells. Then, the volume for 250.000 cells was calculated and added to complete M199 media in order to achieve a total volume of 120 µl, required for seeding a 0.4 microslide (Luer ibiTreat, ibidi^{TM}) prior coated with 1% (w/v) fibronectin (Sigma). Cells left to adhere on the slides overnight at 37 ^{O}C in a 5% incubator. Cells were exposed to flow via the ibidi^{TM} syringe-pump system. The reservoir syringes were sprayed with industrial methylated spirit (IMS) and left to dry out, and the plastic tubing (green and yellow or red, depending on the experimental set up) were autoclaved in order to be sterile before the initiation of each experiment. Depending on the experimental conditions, 6 ml of SBS- 1.5mM Ca^{2+} containing media or SBS- Ca^{2+} free media (Table 2.3) or complete M199 media was added to each reservoir syringe. The ibidi^{TM} software was used to select the perfusion set type and the slide's size. Flow parameters were also defined in the software. The specific flow parameters (magnitude and direction) used in the experiments were: unidirectional High Shear Stress (HSS) of 15 dynes/cm^2, unidirectional Low Shear Stress (LSS) of 5 dynes/cm^2, 1 Hz bidirectional HSS of 15 dynes/cm^2, 1 Hz bidirectional LSS of 5 dynes/cm^2, 0.67 Hz bidirectional HSS of 15 dynes/cm^2 and 2 Hz bidirectional HSS of 15 dynes/cm^2. Depending on the experimental set up, cells were exposed to the above flow conditions for 3 minutes or 24 hours. With the completion of each experiment, the perfusion set was removed from the slides and the reservoir syringes were removed from the fluidic units. The perfusion sets were bleached twice, washed with water and then autoclaved for next use.

2.4.2 Magnetic Tweezers

A platform of magnetic tweezers was developed by Dr. Matthew Bryan (post- doctoral researcher in the Evans lab). The magnetic tweezers are consisted of four poles and magnetic fields were generated by passing electrical current around copper coils wound around a mild steel core and focussed over the sample using the pole pieces either side of the imaging region (Figure 2.1). The profile of the generated magnetic forces (direction and magnitude) can be controlled by a computer software (LabView, National Instruments). In order to determine the magnetic field profile, the Biot-Savart equation was solved over a finite-element mesh covering the imaging region after the computational modelling of the system with the ANSYS software. As the generated forces were proportional to the current supplied to each pole piece, maximum forces of 16 pN were produced either as a unidirectional force (activating a single pole piece) or as a bidirectional force (alternate activation of both pole pieces). The magnetic tweezers are also placed in a heated chamber ($37^{O}C$) around a fluorescence microscope (Figure 2.1), allowing live-cell imaging as the magnetic force is applied.

HUVECs (250.000 cells/dish) were seeded on 1% (w/v) fibronectin (Sigma) or 1% (w/v) gelatized (Sigma) coated 35 mm microdishes (μ-Dish 35 mm, ibidi^{TM}) following the same procedure used to coat 0.4 microslides (Luer ibiTreat, ibidi^{TM}) (see 2.5.1). Following their adhesion on the microdishes, HUVECs were incubated with 15 10^{5}/ml magnetic nanoparticles in serum- free M199 media for 30 minutes at $37^{O}C$ in a 5% incubator. The unbound beads were then washed with 500 μl of SBS imaging buffer (Table 2.2) and the microdishes were placed at the centre of the magnetic tweezers platform. Unidirectional or 1 Hz bidirectional force of ~16 pN was applied onto the cells for the desired period of time.

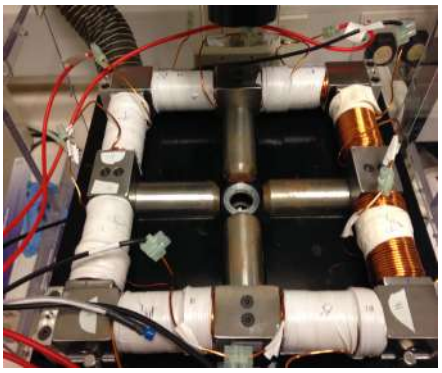
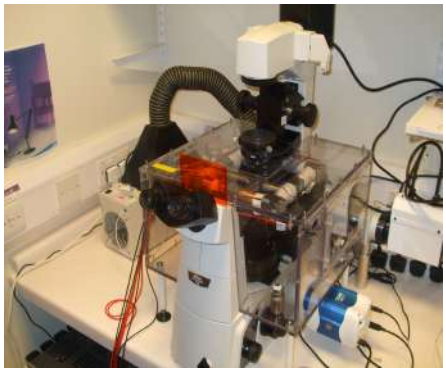
A  B 

Figure 2.1 Magnetic tweezers set up.

(A) Top view of the platform

(B) Perspective view of the whole system: magnetic tweezers, fluorescence microscope, heating supplier.

2.5 Coating of magnetic nanoparticles with antibodies or ligands

2.5.1 Non- covalent coating

Magnetic beads conjugated with goat- anti- mouse IgG or sheep anti- rat IgG (Dynabeads® goat anti- mouse IgG, Dynabeads® sheep anti- rat IgG, Invitrogen) were non- covalently coated with the antibody of interest. To achive this, 20 µl of beads were washed 3 times with 100 µl lysis buffer (1 M Tris, 5 M NaCl, 0.5 M EDTA, 0.3% of TritonX^{TM}). After each wash, a magnet was placed at the site of the Eppendorf tube to form a bead "pellet", in order to remove the excess fluid. 200 µg/ml of the antibody of interest was added to 100 µl of the beads suspension and incubated for 2 hours at room temperature on an Eppendorf rotator. Then, beads were washed with Supplemented PBS (0.1% (w/v) Bovine Serum Albumin, 0.5 M EDTA, pH 7.4) and resuspended in 500 µl serum-free M199 media in a final concentration of 16x 10^6 beads/ml.

2.5.2 Covalent coating

On the first day of the protocol, 20 µl of Dynabeads® M-450 Tosylactivated beads were resuspended and washed twice in 1 ml 0.1 M sodium phosphate Buffer (pH 7.4). 200 µg/ml of the ligand of interest was added to the beads suspension and incubated at room temperature on an Eppendorf rotator overnight.

The next day, a magnet was placed at the site of the Eppendorf tube to form a bead "pellet" and the 0.1 M sodium phosphate buffer was removed.

Then, beads were washed three times with Supplemented PBS (0.1% (w/v) Bovine Serum Albumin, 2 mM EDTA, pH 7.4), each wash for 5 minutes in the cold room (2^{o}C - 8^{o}C). After the final wash, beads were resuspended in Supplemented Tris Buffer (0.2 M Tris, 0.1% (w/v) Bovine Serum Albumin, pH 8.5) and incubated overnight at room temperature on a rotator.

On the final day of the process, a magnet was placed at the site of the Eppendorf tube to form a bead "pellet" and the Supplemented Tris Buffer was removed. Beads were washed three times with Supplemented PBS (0.1% (w/v) Bovine Serum Albumin, 2 mM EDTA, pH 7.4), each wash for 5 minutes in the cold room (2^{o}C - 8^{o}C) and finally diluted in 500 μl serum-free media in a final concentration of 16 10^6 beads/ml.

2.6 Measurement of Calcium responses

2.6.1 Loading of Calcium fluorescent dye

Before the loading of cells with the calcium- sensitive fluorescent dye, 50 μg of Cal-520^{TM} (AAT Bioquest®) was diluted to 5 mM in anhydrous Dimethyl sulfoxide (DMSO, Sigma). HUVECs seeded in 1% (w/v) fibronectin (Sigma) -coated 0.4 microslides (Luer ibiTreat, ibidi^{TM}) or 35 mm microdishes (μ-Dish 35 mm, ibidi^{TM}) were incubated with Cal-520^{TM} (AAT Bioquest®)- Pluronic® F-127 (Invitrogen) diluted in complete M199 media in a ratio of 1 μl:1 μl:1 ml for 75 minutes at 37^{O}C in a 5% incubator. Slides or dishes were kept in foil, as Cal-520^{TM} is light- sensitive. After incubation, cells were washed twice with 150 μl of a Sodium Buffered Saline Ca^{2+} containing media (SBS- 134.3 NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM HEPES, 8 mM Glucose, pH 7.4). For experiments requiring the use of SBS- Ca^{2+} free media in order to deplete the extracellular calcium, 1.5 mM $CaCl_2$ was replaced with 0.4 mM EGTA. To deplete the intracellular calcium stores, cells were incubated with 10 μM Thapsygargin, which inhibits the Ca^{2+} ATPase of the ER (Jena et al., 1997) for 3 minutes.

2.6.2 Live calcium imaging

HUVECs seeded on 1% (w/v) fibronectin (Sigma) -coated 0.4 microslides (Luer ibiTreat, ibidi™) or 35 mm microdishes (µ-Dish 35 mm, ibidi™) were placed on a stage of an inverted epifluorescence microscope embedded in a closed Perspex box and equipped with a supplier of warm air (Figure 2.2). Using a Nikon Eclipse *Ti* camera and a photometrics CoolSnap MYO, 180 consecutive images of the cells were recorded, with each image to be taken every second. After the completion of recording, the sequence of images was stored as stack images, generating a video consisted of 180 frames.

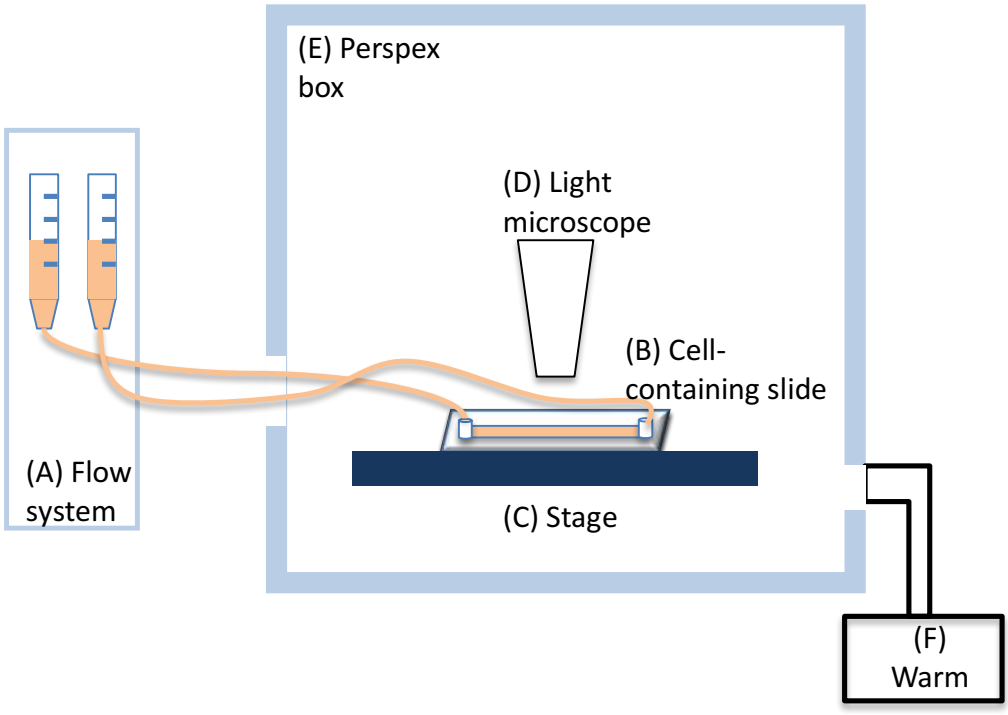


Figure 2.2 The *in vitro* system set up for live calcium imaging.

The ibidi™ flow system (A) was used to generate flow, which was applied onto cells seeded on a slide (B). The slide is placed on a stable stage (C) and under a light microscope (D), which enables live- cell imaging. The microscope and the slide are encased in a Perspex box (E) with a constant supply of warm air (F)

2.6.3 Analysis of Calcium responses

Calcium responses were measured using the intensity (amplitude) of the fluorescent peaks. Using the ImageJ software, up to 50 responding cells were selected in each video. The single cells analysis was used in order to take into account the heterogeneity of the calcium responses. Then, the mean fluorescent intensity of each individual cell was measured for every frame of the 180-frame video. The peak amplitude for each cell was calculated by deducting the minimum intensity value from the maximum intensity value and then dividing by the minimum intensity value (peak amplitude= (max-min)/min) (Figure 2.3B). Peak amplitudes of all selected responding cells were plotted and the median amplitude was calculated (Figure 2.3).

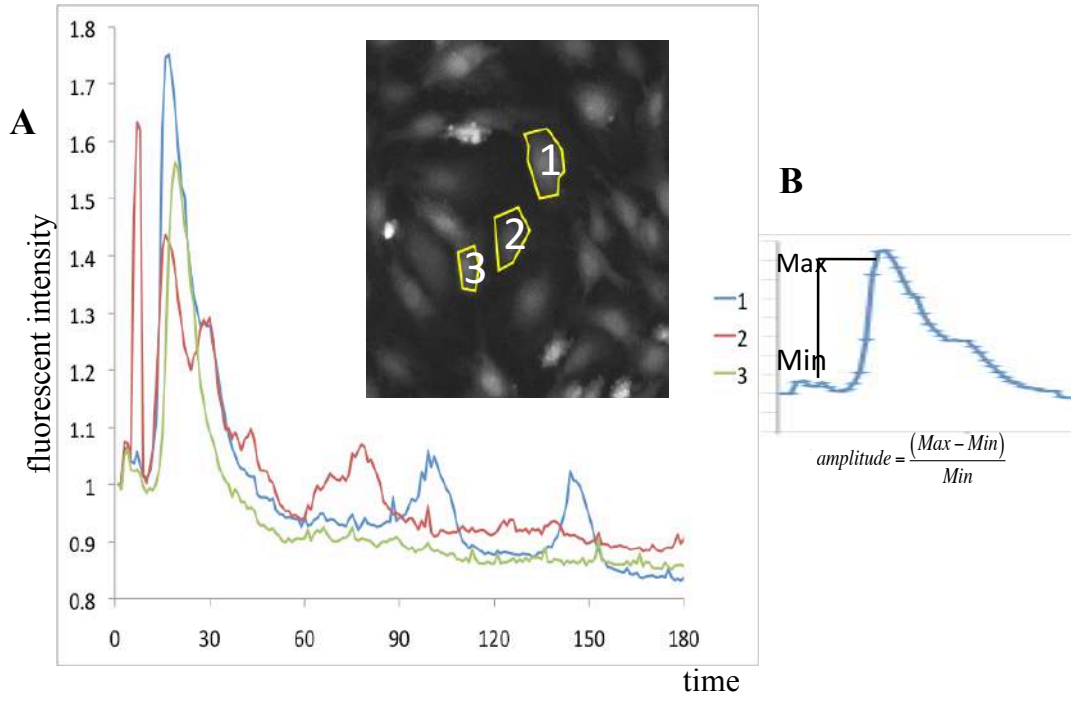


Figure 2.3. Representative graph of calcium peak amplitude of 3 responding cells.

(A) Using ImageJ software, 3 responding cells were selected and the mean fluorescent intensity (y axis) for each cell was measured for every second (x axis).

(B) Equation to calculate the amplitude of each peak.

2.7 Gene expression analysis

2.7.1 RNA isolation

HUVECs seeded on 1% (w/v) gelatinized (Sigma) 6- well plate were washed twice with cold PBS (Sigma). Using the rubber end of a 1 ml syringe, cells were detached from each well. The detached cells were added to a 15 ml tube and centrifuged at 160 RCF for 5 minutes in 21 ^{o}C. 10 µl/ml of β- mercaptoethanol (Sigma) was added to 350 µl RLT- lysis buffer (Qiagen). The cell pellet was resuspended to the β-mercaptoethanol/lysis buffer. By passing the cell suspension through a 21-gauge needle using a syringe, the cell lysate was homogenized. In order to precipitate the nucleic acid, a volume of 70% (v/v) ethanol (Sigma) was added to the cells. Following the manufacturer's protocol, total RNA was extracted using the Qiagen RNeasy^{TM} kit and eluted in 30 µl RNAse- free water (Sigma). RNA purity and concentration were measured spectrophotometrically with a NanoDrop ND- 1000 (Life Technologies, USA). RNA assessment was performed by ND- 1000 v 3.7.0 software.

2.7.2 cDNA synthesis

Using the iScript^{TM} cDNA synthesis kit and according to manufacturer's protocol, RNA at a concentration of 200 ng/ml was reversed transcribed to single strand cDNA. The synthesis was conducted in a thermal cycler (Veriti thermal cycler). The thermal program of the reaction was: step 1 at 25 ^{o}C for 5 minutes, step 2 at 42 ^{o}C for 30 minutes, step 3 at 85 ^{o}C for 5 minutes and step 4 at 4 ^{o}C for 10 minutes.

2.7.3 Quantitative Real – Time PCR (qRT-PCR)

2 ng of cDNA and qRT-PCR master mix according to manufacturer's protocol was added to each well of a 384 well plate. The KAPA SYBR® green detection system was used (KAPA SYBR® FAST qPCR Kit Master Mix (2x) ABI Prism^{TM}). The master mix and the cDNA for each gene of interest were aliquoted in triplicates. Each reaction included KAPA SYBR® FAST qPCR Kit Master Mix (1x) ABI Prism^{TM}, 200 nM of forward primer and 200 nM of reverse primer, 2 ng of cDNA template and nuclease free water in a total volume of 20 µl. The 384 well- plate was sealed with a clear adhesive film and centrifuged at 160 RCF for 1 minute in room temperature.

The qRT- PCR was conducted using the CFX384^{TM} Real- time instrument (BioRad). The thermal program used was: step 1 at 95 ^{o}C for 3 minutes, step 2 at 95 ^{o}C for 5 seconds, step 3 at 60 ^{o}C for 45 seconds x 40 cycles, a final dissociation step at 60 ^{o}C, then 95 ^{o}C for 5 minutes with gradual temperature increase by 0.5 ^{o}C. After the completion of the reaction, non- specific amplification or primer dimers were examined by examination of the primer dissociation curves. Furthermore, primer specificity was measured by analysing the primer-melting curve. The set of primers used are listed in table 2.1

The threshold cycle (CT) for each sample was determined by the Bio- Rad CFX manager 3.0 software. The data from the qRT- PCR was analysed using the comparative CT method (2-ΔΔCT) described by Schmittgen (Schmittgen and Livak, 2008). The ΔCT value was given after the normalization of the CT of each sample with the CT of the housekeeping gene, Hypoxanthine guanine phosphoribosyltranferase (HPRT).

The 2-ΔΔCT calculation was also used to define the mRNA relative fold change for each gene of interest. A relative fold change greater than 1 was considered as increase in gene expression levels compared to the control, whereas a relative fold change lower than 1 was indicative of a decrease in gene expression.

Table 2.1 Sequences of forward and reverse primers for known mechanoreceptors

Target gene	Forward primer (5'->3')	Reverse primer (5'->3')
itgb1	CCCGCGCGGAAAAGATGAA	TGAGCAAACACACAGCAAACT
p2x4	TGGAGTCCCCTAGAAGGTGG	GCCCTTGACCTTGGTCGTAA
p2x7	CACTCGGATCCAGAGCATGAA	CTCACCAGAGCAAAGCAACG
piezo1	ATCGCCATCATCATCTGGTTCCC	TGGTGAACAGCGGCTCATAG
trpc1	TGCGTAGATGTGCTTGGGAG	ATCTGCAGACTGACAACCGT
trpc6	TTAGTGATCGCTCCACAAGC	CGTTGAGTGAGTGGCATTCT
trpm4	CACTGTCCTCTGCATCGACT	CACGTCCTTCATCATCTTGC
trpp1	CTCACGTTCTCAGGCCTCC	GCCAGCACACCAGACTCTTA
trpp2	TTGGAGAAAGAGAGGGAGGA	CGTCATCCTCCTCAGAGTCA
trpv2	GTACCTGAGCAAGACCAGCA	CTCCGTCCTTAAGGTTCAGC
trpv4	CCTCGCTTTATGACCTCTCC	GCGGTTCTCAATCTTGCTG

2.8 Binding affinity assay (cell adhesion assay)

HUVECs from a confluent T75 flask were detached using 5 mM EGTA at 37 ^{O}C in a 5% incubator for 10 minutes. Cells were resuspended in 1000 µl complete M199 media (Table 2.2). Cells' suspension was diluted in Trypan blue in a ratio of 1:10. A Neubauer haemocytometer was used to count the exact number of cells. Then, the volume for 250000 cells was calculated and added to 500 µl of serum free M199 media (Table 2.2). Cells in suspension were incubated with 10 µg/ml P5D2 (Abcam) or 10 µg/ml 12G10 (BioRad) or 10 µg/ml goat- anti- mouse IgG or no antibody for 45 minutes. Microdishes (µ-Dish 35 mm, ibidi^{TM}) were coated with 1% (w/v) fibronectin (Sigma) for 30 minutes. After the incubation of the cells with the different antibodies, the excess fibronectin was removed from the microdishes (µ-Dish 35 mm, ibidi^{TM}) and cells were allowed to attach at 37 ^{O}C in a 5% incubator for 60 minutes. To confirm adhesion of the cells, phase- contrast images were taken with a light microscope at 10x magnification.

2.9 Immunofluorescent staining

2.9.1 Immunofluorescent staining of endothelial cell cultures

Cells were cultured on 1% (w/v) fibronectin (Sigma) 0.4 microslides (Luer ibiTreat, ibidi^{TM}) or 35 mm microdishes (µ-Dish 35 mm, ibidi^{TM}). They were washed twice with PBS (Sigma) and fixed by adding 200 µl 4% formaldehyde for 12 minutes at room temperature. Following the fixation step, cells were washed three times with PBS (Sigma). To permeabilise the cell membrane, 200 µl of 0.1% of TritonX^{TM} (Sigma) in PBS was added and cells were incubated for 15 minutes at room temperature.

To block non- specific binding of the antibodies, 200 µl of 20% (v/v) goat serum (Sigma) was added to each slide/dish and incubated for 30 minutes at room temperature. All primary antibodies used for immunostaining were diluted in 5% (v/v) goat serum (Sigma) to limit non- specific binding. All anti-β1- integrin primary antibodies at a final concentration of 1 µg/ml were added into the cells (200 µl per slide or dish) and incubated at 4 ^{o}C overnight. Depending on the species of the primary antibodies, mouse primary IgG control (Invitrogen 10400C) or rat primary IgG control were used at the same concentration of primary antibodies against the protein of interest.

Cells were washed three times with PBS (Sigma) and 200 µl of either goat anti- mouse AlexaFlour^{TM} 488 (Life Technologies) or AlexaFlour^{TM} 568 (Life Technologies) secondary antibodies at 4 µg/ml were added to each slide/dish and incubated in the dark at room temperature for 1 hour. Cells were washed three times with PBS (Sigma) and 200 µl of nuclear stain DAPI (Life Technologies) at a concentration of 4 µg/ml was added in each slide/dish and incubated for 15 minutes in the dark at room temperature. Cells were washed twice and maintained in PBS (Sigma).
Fluorescence of cells seeded on ibidi^{TM} slides or ibidi^{TM} dishes was visualized with the use of the Inverted wide- field fluorescence microscope LeicaMI4000B at 20x objective.

2.9.2 *En face* immunofluorescent staining of fixed murine aortae

Dissected opened aortic tissue sections were placed in wells of a 96-well plate and washed with PBS (Sigma) with 100 µl twice at 5 minutes' intervals. To block non- specific binding of the antibodies, 100 µl of 20% (v/v) goat serum (Sigma) and 5% (w/v) Bovine Serum Albumin (BSA, Sigma) was added to each well and incubated for 30 minutes at room temperature. All primary antibodies used to stain the tissue sections were diluted in 100 µl of 5% (w/v) BSA (Sigma) at 5 µg/ml.

Aortic tissue sections were incubated with primary antibodies overnight at 4 ^{o}C. Rat primary IgG control was also incubated overnight at 4 ^{o}C, at the same concentration of primary antibodies against the protein of interest. Aortic sections were washed twice with 100 µl of PBS (Sigma) at 5 minutes' intervals and then incubated, depending on the species of the primary antibody, with 100µl of either donkey anti- rat AlexaFlour^{TM} 568 (Life Technologies) or AlexaFlour^{TM} 488 (Life Technologies) secondary antibody diluted at 1:300 in 5% (w/v) BSA (Sigma) overnight at 4 ^{o}C.

Tissue sections were washed twice with 100 µl of PBS (Sigma) at 5 minutes' intervals and then stained with CD31 conjugated with AlexaFlour^{TM} 488 (Bio Legend), diluted in a final concentration of 1:100 in 5% (w/v) BSA (Sigma), used as an endothelial cell specific marker. The 96-well plate was covered in foil and tissue section were incubated for 4 days at 4 ^{o}C.

Tissue sections were washed three times with 100 µl of PBS (Sigma) and TO- PRO- 3 Iodine (Life technologies) was used as a nuclear stain, diluted in 1:300 in PBS (Sigma) and incubated for 1 hour at room temperature.

The stained aortic sections were mounted on a microscopy slide in 10 µl Prolong® gold anti-fade reagent (Invitrogen), using metal tweezers and covered with a glass cover slip. In order to maintain tissue fixation, slides were placed overnight at 4 ^{o}C under a load.

Confocal microscopy was performed using a 488 laser to image Alexa Fluor 488, a 543 laser to image Alexa Fluor 568 and a 633 laser for the TO- PRO- 3 Iodine nuclear stain.

2.9.3 Confocal Microscopy

Transmitted light at 20x magnification was used to view the specimens and identify particular regions using anatomical markers. Lasers of the confocal microscope (LSM510, Zeiss) were set to detect excitation of specific fluorophores. An Argon laser was set at 6.1 Å to detect the excitation of fluorescence emitted at 488 nm, a 542 laser to detect the excitation of fluorescence emitted at 568 nm and a 633 laser to detect excitation of fluorescence emitted at 642/661 nm. The data depth of 12 bits with averaging was set at 8 and the frame scan mode was selected at 1024 pixels. The samples were first scanned with the detector for the endothelial specific marker, the pin- hole adjusted accordingly to obtain optimal fluorescence level and was set to be identical for each channel. Samples stained with IgG control were examined to ensure that did not emit any background fluorescence and these settings were also maintained to image the samples of interest. To obtain a z- stack of whole endothelial cells, the specimen was scanned at the z- axis marking the apical surface of the cells as the first slice and the basal surface as the last slice. Z- stack images were acquired at 0.5 μm steps, which is the limit of the resolution of the confocal microscope.

2.10 Statistical analysis of Data

In order to test statistical significance of two experimental conditions, a two tailed paired t- test was used. In experiments with more than two experimental conditions, a one-way ANOVA was used. Finally, statistical analysis of experiments with one measurement variable and with two or more nominal variables, was performed with the two- way ANOVA method.

In order to define the number of experimental repeats, power calculations based on published literature and preliminary data were performed for a power of 0.8 and an alpha of 0.05. For example, preliminary data from calcium response experiments revealed a baseline median amplitude of 4 ± 1.125. Based on president of the literature (Matthews et al., 2010), a calcium response of 50% was deemed significant. These power calculations revealed a minimum sample size of 5 per condition to achieve sufficient power.

Table 2.2 Growth media constituents

Media	Serum Contents	Antibiotics	Supplements
Complete M199 growth medium	20% FCS	2 U/ml Pen-Strep. 0.025 µg/ml Amphotericin B.	0.05 mM L-Glutamine. $5x10^{-3}$ µg/ml endothelial growth supplement. 0.09 µg/ml heparin
Serum- free M199 growth medium	5% FCS	2 U/ml Pen-Strep. 0.025 µg/ml Amphotericin B.	0.05 mM L-Glutamine. $5x10^{-3}$ µg/ml endothelial growth supplement. 0.09 µg/ml heparin

Table 2.3 SBS buffer for Ca^{2+} (1.5 mM Ca^{2+}, pH 7.4) signaling experiments.

Component	Concentration (mM)	g/l	g/500ml	Ca^{2+} free version (g/ 500ml)
NaCl	134.3	7.85	3.9	3.9
KCl	5	0.3728	0.1864	0.18
$MgCl_2$	1.2	1.2 ml of 1 M	600 µl of 1 M	600 µl of 1 M
$CaCl_2$	1.5	1.5 ml of 1 M	750 µl of 1 M	Replace with EGTA 0.4 mM = 0.075 g/500 ml
HEPES	10	2.3830	1.1915	1.19
Glucose	8	1.4413	0.7206	0.72

Chapter 3: Different flow patterns induce divergent calcium responses in endothelial cells

3.1 Introduction

An increase in intracellular calcium levels is one of the initial endothelial responses to shear stress (Ando et al., 1988, Gautam et al., 2006). Calcium responses increase linearly with the shear stress magnitude in steady flow (Yamamoto et al., 2003). Calcium is a key second messenger molecule that has been shown to play a role in synthesis and release of various bioactive molecules, such as von Willebrand factor and nitric oxide (Elies et al., 2011, Devika and Jaffar Ali, 2013, Ando and Yamamoto, 2013). Therefore, calcium is involved in multiple activities in endothelial cells, including migration, proliferation, apoptosis and cytoskeletal reorganisation (De Bock et al., 2013, Moccia et al., 2014). As a consequence, calcium signalling regulates several cellular functions involved in both physiology and pathology of the endothelium. The majority of endothelial cell functions are influenced by both the magnitude and direction of shear stress. However, it is not clear whether calcium responses in endothelial cells also depend on the direction and frequency of flow.

The calcium signalling of endothelial cells may originate from two different sources (Kwan et al., 2003, Liu et al., 2011). Calcium can be released from the intracellular calcium stores of the endoplasmic reticulum or enter the cell, as an influx, from mechanosensitive ion channels on the plasma membrane. Extensive study of calcium signalling in endothelial cells under flow has reported that different molecules are involved in the regulation of these processes, with phospholipase C having a central role in calcium release from the intracellular stores and transient receptor potential channels (TRP), piezo- 1 or P2X receptors on the plasma membrane to control calcium influx of extracellular origin (Berridge, 1993, Ando and Yamamoto, 2013).

Activation of phospholipase C leads to hydrolysis of the lipid precursor phosphatidylinositol- 4,5- biphosphate (PIP_2) to generate IP_3 and diacylglycerol (DAG) (Kadamur and Ross, 2013). Production of IP_3 leads to its binding to the IP_3 receptors, located on the surface of the endoplasmic reticulum. IP_3 receptors are calcium channels, which pump calcium from the endoplasmic reticulum to the cytoplasm (Bourguignon et al., 1994). On the other hand, calcium channels located on the surface of the endothelial cells, can pump calcium into the cells after their stimulation with mechanical forces or small molecules, such as ATP (Yue et al., 2015, Gerhold and Schwartz, 2016). These two distinct mechanisms, controlling the origin of calcium in endothelial cells may cooperate and the stimulation of the one affects the function of the other, for instance the release of calcium from the intracellular stores may lead to opening of the TRP channels and subsequent extracellular calcium influx (Clapham, 2003). It is not known yet if the direction of flow can affect the origin of calcium signalling in endothelial cells.

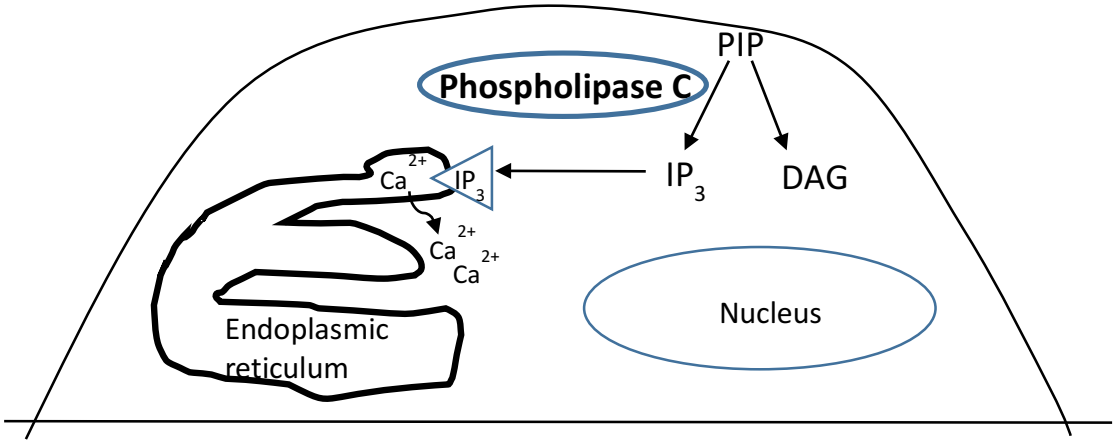


Figure 3.1 Schematic representation of Phospholipase C regulated calcium release from the endoplasmic reticulum

Phospholipase C mediates the hydrolysis of lipid precursor phosphatidylinositol-4,5- biphosphate (PIP_2) to generate IP_3 and diacylglycerol (DAG). IP3 binds to IP3 receptors, located on the surface of the endoplasmic reticulum, promoting calcium release into the cytoplasm.

The flow pattern governs several pathophysiological conditions. *In vivo* studies of atherosclerosis in mice, with the insertion of a tapering cast to the mouse carotid artery, have shown that the development of atherosclerotic lesions follows a flow- dependent spatial distribution (Cheng et al., 2005). The cast creates a fixed geometry, whereby the region downstream of the cast is subjected to oscillatory shear stress, the vessel area within the cast is exposed to high shear stress and upstream region is exposed to low shear stress. Atherosclerotic lesions developed in response to both low and oscillatory shear stress using *apoE*$^{-/-}$ mice fed an atherogenic diet. Furthermore, plaques with characteristics of instability, such as thin fibrous cap and inflammation developed under low shear whereas plaques with a stable fibrotic phenotype developed at the oscillatory shear region. On the contrary features of atheroprotective phenotype, such as elevation of eNOS expression, was observed in regions of the cast with high shear stress (Cheng et al., 2006). However, we do not know how endothelial cells sense and respond differently to distinct flow patterns.

In order to measure the effects of different flow patterns on calcium dynamics and identify the mechanisms of calcium accumulation in the cytosol, live- cell calcium imagining was used in endothelial cells under flow of different magnitude and direction.

Hypothesis

We hypothesize that endothelial cells convert different flow patterns (unidirectional vs bidirectional flow) into distinct calcium responses.

Aims

1. Determine if calcium responses in endothelial cells are flow- pattern dependent (unidirectional vs bidirectional flow at different frequencies)
2. Identify the source and mechanism of calcium responses in endothelial cells under different flow patterns

3.2 Results

3.2.1 Distinct flow patterns induce calcium responses in endothelial cells

Our first aim was to determine if calcium signalling is induced in endothelial cells by the application of different flow patterns. In order to test this, static HUVECs loaded with a calcium- sensitive fluorescent dye were exposed, using the ibidi™ pump system, to either unidirectional or 1 Hz bidirectional flow of 15 dynes/cm^2 (High Shear Stress- HSS) or 5 dynes/cm^2 (Low Shear stress- LSS) for 3 minutes. The dynamics of calcium signaling were assessed by fluorescent microscopy of live cells. As a control, the calcium signaling of HUVECs under static conditions was also measured for 3 minutes in order to quantify the baseline calcium responses (Figure 3.2 A and B).

Flow caused rapid accumulation of Ca^{2+} in endothelial cells. I measured the peak amplitude of the calcium response, which can be translated as the initial response of the cells to flow. It was found that both unidirectional and 1 Hz bidirectional flow patterns enhance calcium signaling. The amplitude of the calcium peak was induced in both flow conditions (Figure 3.2 C). Furthermore, the application of flow of either high or low shear stress induced calcium response in HUVECs but there was little difference between the response to low or high shear stress.

These data indicate that endothelial cells mount calcium signaling in response to unidirectional and bidirectional flow.

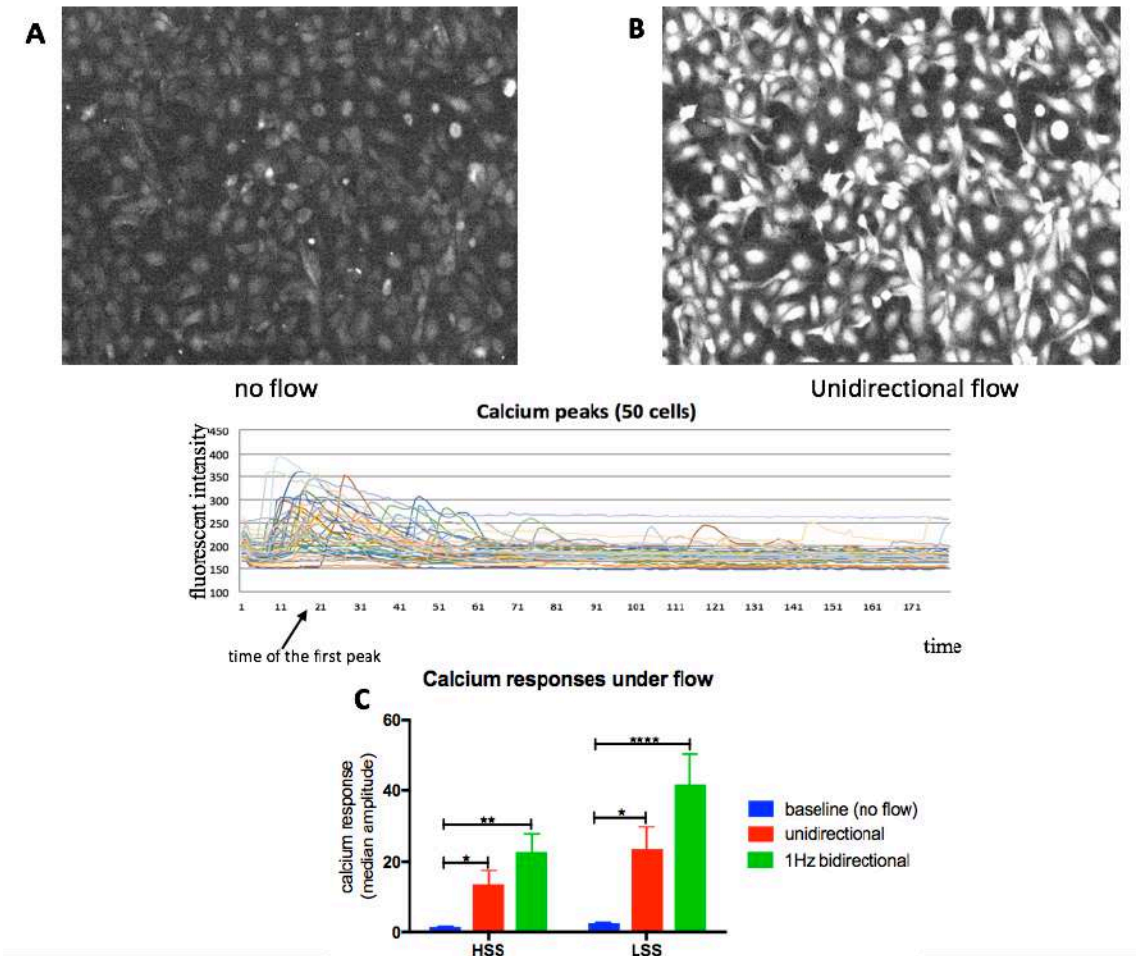


Figure 3.2 Distinct flow patterns induced calcium signalling in HUVECs

HUVECs were loaded with calcium fluorescent dye and exposed to flow for 3 min, using the ibidi^{TM} system. Calcium responses in real time were recorded using fluorescent microscopy. Representative images showing calcium response in HUVECs under no flow (A) or unidirectional flow of 15 dynes/ cm^2 (B). The representative line graph shows individual calcium responses of 50 cells in relation to time. The first calcium response occurs around 15 seconds (indicated by the arrow) after the initiation of flow.

Quantification of calcium responses in HUVECs under flow of different direction [no flow (blue), unidirectional (red) and 1 Hz bidirectional (green)] and different magnitude [(High shear stress (15 dynes/cm^2, HSS) and Low Shear Stress (5 dynes/cm^2, LSS)] for 3 minutes. Calcium responses were analysed by selecting up to 50 responding cells per experiment. The amplitude of the calcium response was analysed by measuring the median peak amplitude of the responding cells (C).
(n=4, * indicates p=<0.05, ** indicates p=<0.01, **** indicates p=<0.0001 using a two- way ANOVA. Values are ± SEM)

3.2.2 Endothelial cells convert specific frequencies of shear stress into distinct calcium responses

Acute application of flow induced calcium signaling in endothelial cells (Figure 3.2). These responses were enhanced in cells under either high shear stress or low shear stress, suggesting that the magnitude of shear stress does not affect the initial calcium response within the physiological range. However, bidirectional flow can have different frequencies. Therefore, we examined whether endothelial cells can convert different applied frequencies of bidirectional flow into specific calcium signals.

In order to address this aim, static HUVEC loaded with a calcium-sensitive fluorescent dye were exposed to 0.67 Hz, 1 Hz or 2 Hz of 15 dynes/ cm^2 bidirectional flow for 3 minutes (Figure 3.3 A). The quantification of the peak amplitude of calcium signaling showed that endothelial cells can distinguish between different applied frequencies of bidirectional flow (Figure 3.3 B). The application of 0.67 Hz and 2 Hz of bidirectional flow failed to trigger a significant calcium response compared to no flow conditions. On the contrary, application of 1 Hz bidirectional flow induced a significant increase of calcium signaling compared to either no flow or 0.67 Hz and 2 Hz bidirectional flow conditions. As a result, HUVECs do not respond linearly with the duration of each cycle of bidirectional flow.

These data indicate that endothelial cells are sensitive to oscillation frequency rather than oscillation *per se*.

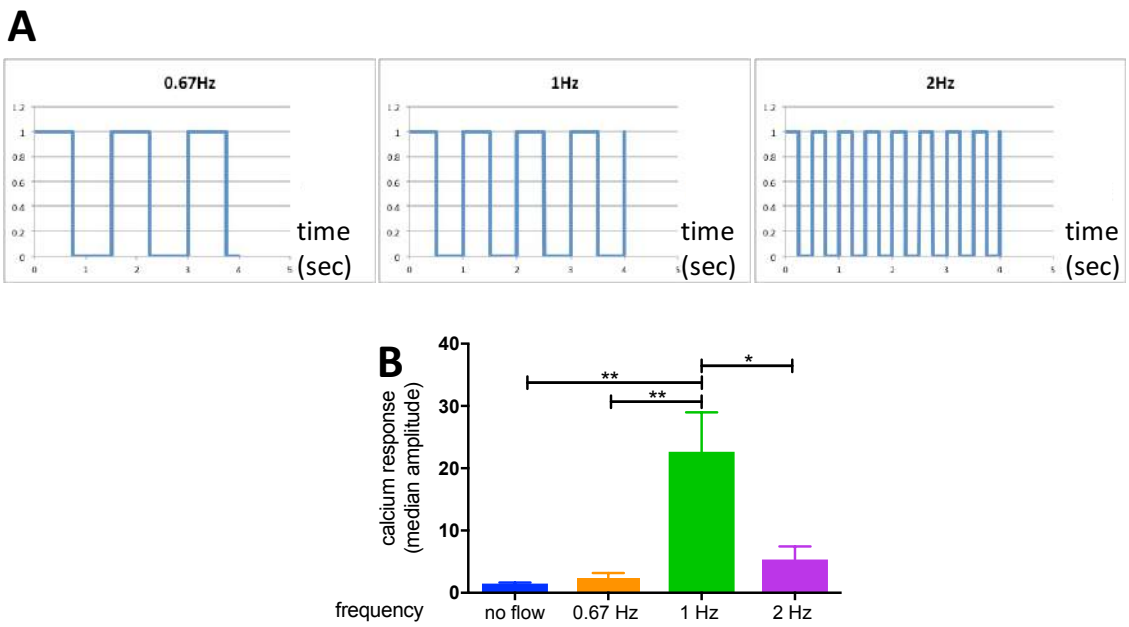


Figure 3.3 Calcium responses in endothelial cells were depended on oscillation frequency

To generate the desired flow patterns, the ibidi™ pump system was used. Flow profiles of 0.67 Hz, 1 Hz and 2 Hz in relation to time (A). High and low states represent forward and reverse flows, respectively.

Calcium responses in HUVECs under different frequencies of bidirectional flow [no flow (blue), 0.67 Hz (orange), 1 Hz (green), 2 Hz (pink)] of 15 dynes/ cm^2 for 3 minutes. The calcium responses were recorded using fluorescent microscopy of live cells. Calcium responses of HUVEC under static conditions were assessed as a control. 1 Hz bidirectional flow induced calcium signalling in HUVECs compared to no flow. The other frequencies failed to induce a significant calcium response.
(n=4, * indicates p=<0.05, ** indicates p=<0.01 using a one- way ANOVA with Tukey's test for multiple comparisons. Values are ± SEM)

3.2.3 Calcium responses in endothelial cells under distinct flow patterns have different mechanisms

I previously demonstrated that endothelial cells mount specific calcium signaling in response to different frequencies of bidirectional flow (Figure 3.3). As calcium can either originate from the intracellular stores of the endoplasmic reticulum or have an extracellular origin (Figure 3.4), it was examined whether the application of distinct flow patterns affects these two calcium sources differently.

To test whether the calcium responses originate from the intracellular stores, static HUVECs were treated with 10 μM of Thapsigargin for 3 minutes prior to the application of flow. Thapsigargin is an irreversible inhibitor of the sarco/endoplasmic reticulum calcium ATPase (SERCA). SERCA keeps the cytoplasmic levels of calcium low by pumping calcium into the sarco/endoplasmic reticulum (Tran et al., 2000). Thus, inhibition of SERCA with Thapsigargin leads to calcium diffusion from the endoplasmic reticulum into the cytoplasm. Addition of Thapsigargin to static HUVECs loaded with a calcium fluorescent dye led to a rapid calcium response (Figure 3.5 A), confirming the depletion of intracellular calcium stores.

Immediately after the treatment with Thapsigargin, HUVECs were exposed to either unidirectional or 1 Hz bidirectional flow of 15 dynes/cm^2 for 3 minutes and the calcium response was recorded using fluorescent microscopy of live cells. As a control, calcium signaling of untreated HUVECs under unidirectional or no flow was also recorded for 3 minutes. It was found that prior treatment with Thapsigargin totally abolished the calcium response in response to unidirectional flow (Figure 3.5 B). On the contrary, HUVECs pre- treated with Thapsigargin but then exposed to 1 Hz bidirectional flow showed a decreased but not absolute reduction of the calcium signaling (Figure 3.5 B).

On the other hand, to examine whether calcium responses under different flow patterns have an extracellular origin, the extracellular calcium was chelated using EGTA. HUVECs loaded with a calcium fluorescent dye were exposed to unidirectional or 1 Hz bidirectional flow of 15 dynes/cm^2, with the EGTA- Ca^{2+} free- containing buffer, and calcium signaling was recorded for 3 minutes using fluorescent microscopy. The calcium responses of cells under 1 Hz bidirectional flow with normal Ca^{2+}-containing buffer or no flow were also recorded as a control. The calcium response of HUVECs under either unidirectional or 1 Hz bidirectional flow with the EGTA- Ca^{2+} free- containing buffer was altered but not significantly reduced compared to untreated cells (Figure 3.5 B).

Moreover, as a positive control both the intracellular calcium stores were depleted and calcium from the extracellular buffer was removed (Figure 3.5 C). This was achieved by treating HUVECs with Thapsigargin for 3 minutes and then exposed to unidirectional or 1 Hz bidirectional flow of 15 dynes/cm^2 for 3 minutes with a EGTA- Ca^{2+} free- containing buffer. It was found that pre- treatment of HUVECs with Thapsigargin and then application of unidirectional or 1 Hz bidirectional flow with EGTA- Ca^{2+} free-containing buffer totally abolished calcium responses in both flow conditions (Figure 3.5 C).

These data suggest that calcium signaling in HUVECs under unidirectional flow originate from the intracellular stores of the endoplasmic reticulum, whereas the calcium response in cells under 1 Hz bidirectional flow involves both the intracellular calcium stores and extracellular calcium.

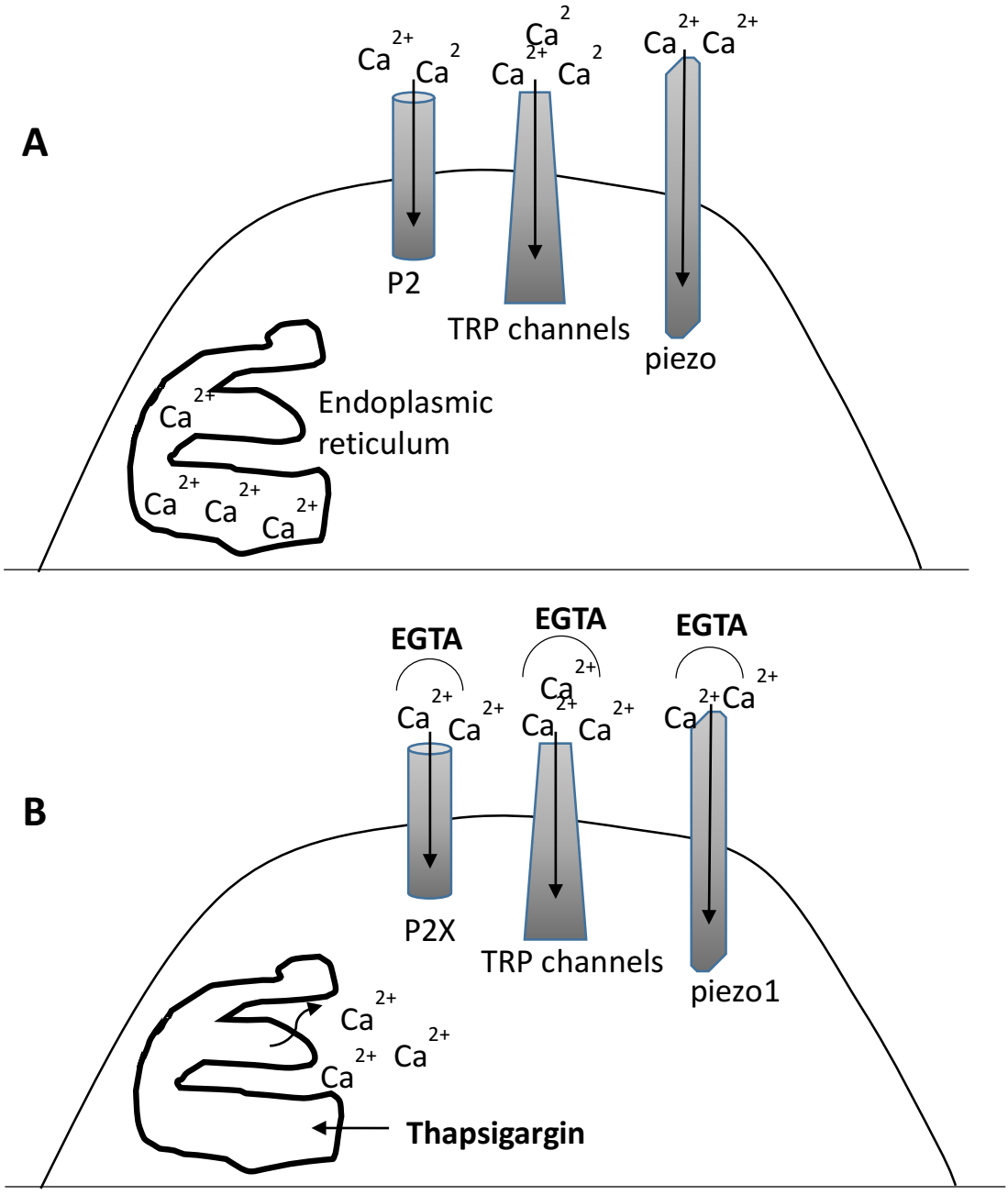
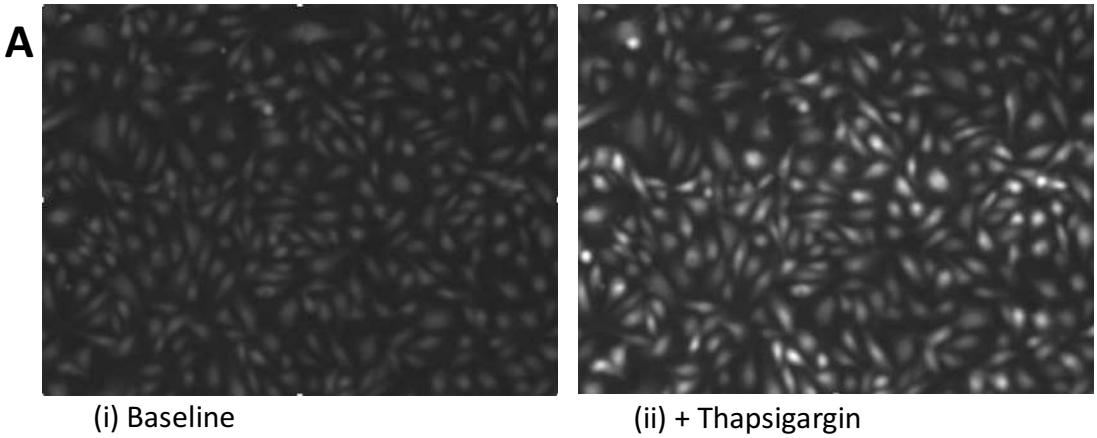
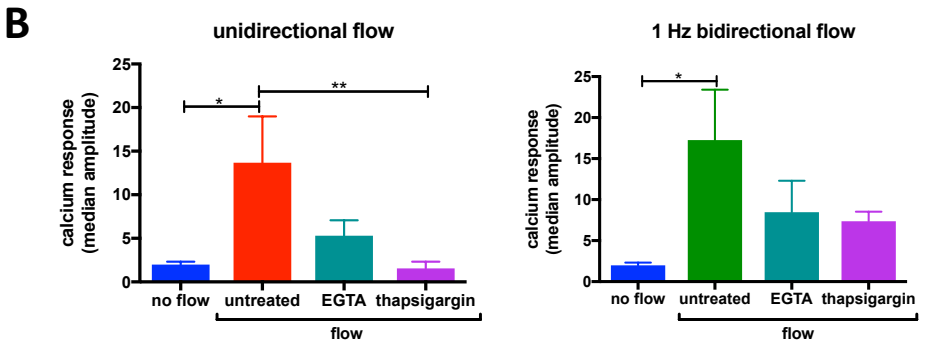


Figure 3.4 Schematic representation showing the effect of Thapsigargin and EGTA on calcium responses

(A) Calcium signalling in endothelial cells can either have extracellular (influx through calcium channels) or intracellular (endoplasmic reticulum stores) origin.

(B) Treatment with Thapsigargin leads to depletion of intracellular calcium stores, whereas chelation of extracellular calcium with EGTA blocks calcium influx through the calcium channels of the plasma membrane.


A
(i) Baseline
(ii) + Thapsigargin


B
unidirectional flow
1 Hz bidirectional flow
calcium response (median amplitude)
25
20
15
10
5
0
*
**
no flow
untreated
EGTA
thapsigargin
flow

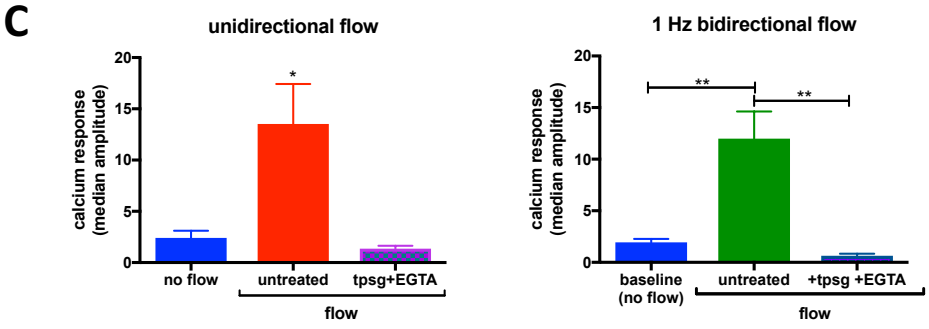

C
unidirectional flow
1 Hz bidirectional flow
calcium response (median amplitude)
20
15
10
5
0
*
**
no flow
untreated
tpsg+EGTA
baseline (no flow)
+tpsg +EGTA
flow

Figure 3.5 Origin of calcium signalling in endothelial cells under different flow patterns

(A) To confirm the action of Thapsigargin, the calcium response of static HUVECs loaded with a calcium fluorescent dye was recorded for 3 minutes before (i) and immediately after (ii) the addition of Thapsigargin using fluorescent microscopy of live cells.

(B) Cells were left untreated (red for unidirectional flow, green for 1 Hz bidirectional flow), or treated with EGTA- Ca^{2+} free- containing buffer (dark blue) or with Thapsigargin (purple). Baseline (no flow) conditions are shown as light blue. Pre-treatment with thapsigargin in HUVECs under unidirectional flow significantly reduced calcium responses.
(n=5, * indicates p=< 0.05, ** indicates p=< 0.01 using a one- way ANOVA test, with Tukey's test for multiple comparisons. Values are ± SEM)

(C) HUVECs were left untreated (red for unidirectional flow, green for 1 Hz bidirectional flow) or pre- treated with thapsigargin (tpsg) and then exposed to flow with EGTA- Ca^{2+} free- containing buffer (dark blue and purple bar). Baseline (no flow) conditions are shown as light blue. Pre-treatment of HUVECs with thapsigargin and exposure to either unidirectional or 1 Hz bidirectional flow using a EGTA- Ca^{2+} free- containing buffer, totally abolished calcium responses.
(n=3, * indicates p=< 0.05, ** indicates p=< 0.01, *** indicates p=< *** p<0.001, **** indicates p=< 0.0001 using a one- way ANOVA test, with Tukey's test for multiple comparisons. Values are ± SEM)

3.2.4 Phospholipase C regulates calcium responses in endothelial cells under unidirectional flow

I observed that intracellular calcium stores play a significant role in the calcium signaling of endothelial cells under unidirectional flow (Figure 3.5 B). To further elucidate the mechanism involving the calcium release from the endoplasmic reticulum, a molecule crucial for its regulation, called Phospholipase C, was examined. Shear stress is known to activate Phospholipase C and its activation leads to production of IP_3, which binds to specific receptors of the endoplasmic reticulum leading to subsequent release of calcium into the cytoplasm. Therefore, in order to examine the role of Phospholipase C in calcium signalling of cells under unidirectional flow, Phospholipase C was inhibited pharmacologically using U- 73122.

HUVECs loaded with a calcium- sensitive fluorescent dye were treated with the phospholipase inhibitor or left untreated and then exposed to unidirectional flow. Analysis of the first peak amplitude showed that HUVECs prior to treatment with Phospholipase C inhibitor had a significantly decreased calcium response (~ 50%) when exposed to unidirectional flow compared to untreated cells (Figure 3.6). However, pre-treatment of HUVECs with Phospholipase C inhibitor did not abolish the calcium responses under flow completely, indicating that other molecules are also involved in this process.

This finding indicates that Phospholipase C regulates the release of calcium in endothelial cells exposed to unidirectional flow.

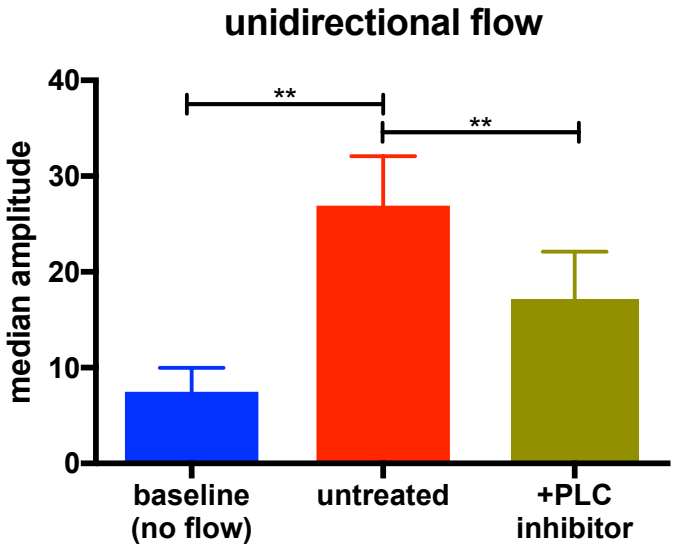


Figure 3.6 Phospholipase C regulated calcium responses in endothelial cells under flow

HUVECs loaded with a calcium fluorescent dye were incubated for 15 minutes with the U- 73122 Phospholipase C inhibitor (gold) and then exposed to unidirectional flow of 15 dynes/ cm^2 for 3 minutes. As a control, HUVECs were left untreated (red) and exposed to the same flow condition or no flow (blue) for 3 minutes. Calcium responses were recorded using a fluorescent microscope. Quantification of the median amplitude showed that inhibition of Phospholipase C led to a reduced calcium response under unidirectional flow.
(n=4, * indicates p=< 0.05, ** indicates p=< 0.01, using a one- way ANOVA test, with Tukey's test for multiple comparisons. Values are ± SEM)

3.3 Discussion

3.3.1 Conclusions

- Acute application of both high and low shear stress of unidirectional or 1 Hz bidirectional flow induce calcium signaling in endothelial cells
- Different frequencies of bidirectional flow trigger distinct calcium responses in endothelial cells
- The mechanism of calcium signalling in endothelial cells depends on the flow pattern, with unidirectional flow to induce calcium release from the inner stores and bidirectional flow to trigger both release from the inner stores and influx from the extracellular media
- Phospholipase C regulates intracellular calcium signalling in endothelial cells under unidirectional flow

3.3.2 Discussion and Future work

Flow patterns and Ca^{2+} sources

This study is the first to test the calcium signalling in endothelial cells exposed to flow patterns of different magnitude, direction and frequency. In addition, these experiments showed for the first time that the origin of calcium signalling depends on the direction of flow.

The investigation of the mechanism of calcium responses in endothelial cells under flow led to the conclusion that calcium signalling has a different source depending on the applied flow pattern. Depletion of intracellular stores abolished the calcium responses to unidirectional flow but had only partial effects on cells exposed to bidirectional flow. The depletion of intracellular calcium stores of the endoplasmic reticulum (ER) with thapsigargin may lead to store operated calcium entry (SOCE) via calcium released- activated channels (Tran et al., 2000).

These calcium channels are located at the plasma membrane and are activated when calcium is depleted from the ER, allowing extracellular calcium to enter the cells and slowly replenish the intracellular stores. It is possible that this mechanism is activated more extensively in cells under bidirectional flow than in cells under unidirectional flow and this is the reason why even after the depletion of intracellular stores with thapsigargin, a calcium response was observed in cells under bidirectional flow. An additional explanation could include the role of mitochondria in the regulation of calcium responses in endothelial cells. Mitochondria are another crucial container of intracellular calcium (Wood and Gillespie, 1998). Studies have demonstrated that exposure of endothelial cells to oscillatory shear stress increased intracellular O_2^- as a consequence of sustained mitochondrial activation (De Keulenaer et al., 1998). Therefore, it is possible that the observed calcium response under bidirectional flow when calcium stores of the ER are depleted with thapsigargin, is triggered by calcium release from the mitochondria. The involvement of either the SOCE mechanism or the mitochondrial calcium stores was not tested further in the current study, but could be an interesting field for future work. Finally, one explanation for the different origin of calcium responses under distinct flow patterns can rely on the function of different Ca^{2+}- regulating mechanoreceptors. Studies have shown that mechanoreceptors can respond to different mechanical forces (Baeyens et al., 2014). As a result, specific mechanoreceptors can be activated and transducing signals under unidirectional flow, resulting to release of calcium from the ER but not influx from the extracellular media, whereas other mechanoreceptors can function under bidirectional flow only, leading to both intracellular calcium release and extracellular calcium influx. The latter case could include mechanosensitive calcium channels, such as TRP channels or P2X channels. Therefore, the investigation of the role of specific mechanoreceptors on regulation of distinct calcium signalling in endothelial cells under flow is of great importance.

Oscillatory frequency and Ca^{2+} responses

This project is the first to investigate the effect of different frequencies of bidirectional flow on calcium dynamics of endothelial cells. Interestingly, it was found that only 1 Hz bidirectional flow induced calcium signalling whereas 0.67 Hz and 2 Hz bidirectional flow failed to trigger significant calcium responses. This finding is of great importance as the normal heartbeat has also a frequency of 1- 1.67 Hz (Kim et al., 2007). In addition, a study of Feaver showed haemodynamic frequency harmonics is a critical regulator of inflammatory endothelial responses, leading to Nf-kB activation (Feaver et al., 2013). As endothelial cells have specific mechanoreceptors on their surface that can sense mechanical forces, there may be an optimal frequency of bidirectional flow that can be transmitted into the cell as a specific biochemical signal, in this case as calcium response. Several studies have shown that endothelial cells have a range of different mechanoreceptors, which differ in structure and function. These differences in the molecular structure could explain the response of endothelial cells to specific frequencies. Future work could elucidate the mechanisms by which specific mechanoreceptors sense distinct mechanical forces by exploring the structural rearrangements of these molecules under force.

Signalling to Ca^{2+} under unidirectional flow

The finding that calcium responses under unidirectional flow have an intracellular origin was further investigated by exploring the role of phospholipase C. Its inhibition with U-73122 led to decreased calcium responses under unidirectional flow. Although U-73122 is a well- described agent for inhibition of phospholipase C, its specificity is not that well defined (Nofer et al., 1997, Leitner et al., 2016).

Phospholipase C is classified to six isotypes (β, γ , δ, ε, ζ, η) in mammals according to their structure (Rhee, 2001, Nomikos et al., 2005, Hwang et al., 2005). In order to identify which specific isoforms play a role in regulation of calcium signalling under unidirectional flow, future work could include the use of specific siRNAs targeting different isoform each time. The silencing of specific isoforms would identify the exact isoforms responsible for calcium signalling under flow. Finally, the investigation of which mechanoreceptors crosstalk with phospholipase C in endothelial cells under unidirectional flow, could elucidate the mechanisms of how endothelial cells sense and respond to distinct flow patterns.

Role of Ca^{2+} in atheroprotection and atherosusceptibility

The application of unidirectional or 1 Hz bidirectional flow of either high or low shear stress induced calcium responses in HUVECs. However, there were no significant differences in calcium dynamics amongst different applied flow patterns, with all patterns to cause a similar calcium response in HUVECs. Several studies have shown that unidirectional flow of high shear stress and bidirectional flow of low shear stress have distinct effects on endothelial cells' pathophysiology. To be more specific, exposure of endothelial cells to unidirectional flow of high shear stress is known to induce an anti- inflammatory and anti- apoptotic response, by inducing the expression of molecules such as KLF2, KLF4 and eNOS. Therefore, high shear unidirectional flow is considered atheroprotective. On the contrary, the application of bidirectional (oscillatory) flow of low shear stress promotes inflammatory and apoptotic responses in endothelial cells, by upregulation of molecules such as VCAM, ICAM- 1 and NF- kB. As a result, low shear bidirectional flow is thought to be atheroprone.

The distinct mechanisms involved in calcium signalling in endothelial cells under different flow patterns may also be associated with some processes involved in the initiation and progression of atherosclerosis.

To be more specific, the calcium release from the intracellular stores and the molecules involved in this process, which are activated from unidirectional flow (such as phospholipase C), only may activate downstream signalling pathways promoting anti- inflammatory, anti-apoptotic responses, whereas the activation of calcium channels on the cell membrane under bidirectional flow, which leads to influx of calcium with extracellular origin, may lead to activation of pathways related to inflammatory responses. Studies have shown that calcium influx through endothelial TRP channels, such as TRPV1, enhance adhesion of monocytes on endothelial cells, an early event in the process of atherosclerosis (Himi et al., 2012). In addition, other studies suggest that calcium influx through endothelial TRPC3 regulates expression of inflammatory molecules, such as VCAM-1 and NF- kB (Smedlund et al., 2010). Further studies are required to elucidate the link amongst flow direction, calcium signalling source and atherosclerosis.

Limitations of the study

First of all, the type of the endothelial cells used in the experiments seem to play an important role. Different endothelial cell types, depending on their anatomical location, are under the effect of different haemodynamic shear stress patterns. For instance, a study of Jow has shown that flow regulates calcium responses in human aortic endothelial cells (HAECs) but not in human capillary endothelial cells (Jow and Numann, 1999). The experiments of my study were performed with static HUVECs exposed to acute application of flow. These cells, with a venous origin, are physiologically exposed to shear stress magnitudes of 1- 6 dynes/ cm^2 (Malek et al., 1999). As a consequence, HUVECs have not experienced shear stress values of 15 dynes/ cm^2 used in this set of experiments.

Therefore, HUVECs may have a “plateau” of the calcium signalling and acute application of flow with a shear stress magnitude above 6 dynes/ cm^2 may not increase calcium responses proportionally. Future work could include the study of other endothelial cell types, such as HAECs or human coronary artery endothelial cells (HCAECs). In addition, it would be interesting to pre- expose endothelial cells to flow for a long period of time (up to 72 hours) and then test the effect of acute application of different flow patterns on calcium dynamics. The long exposure of endothelial cells to flow can alter the expression levels of flow- regulated genes that could potentially contribute to calcium responses. Although, the experiments of this project included the exposure of static HUVECs to acute flow, there are some processes where acute application occurs, such as the onset of flow during embryogenesis or coronary artery bypass graft surgeries. Finally, the calcium responses were recorded *in vitro*. Future experiments could test calcium signalling under distinct flow patterns *in vivo,* with the use of an experimental model such as zebrafish, which has been previously used to examine effect of flow on vascular biology and calcium dynamics (Quaife et al., 2012, Goetz et al., 2014).

In summary, the first limitation of the performed experiments is the acute application of flow, which does not mimic the chronic blood flow in the human body. However, the study of calcium responses, an acute first response to flow, is important to understand the rapid responses of endothelial cells under flow. Furthermore, the use of HUVECs only may limit the study of endothelial cell behaviour under flow, as different types of endothelial cells respond differently to haemodynamic forces. The use of other endothelial cells types, especially with an arterial origin such as HCAECs, would broaden our knowledge regarding the calcium responses under flow.

Finally, the use of pharmacological agents, such Thapsigargin and Phospholipase C inhibitor, may trigger off- target effects. The use of siRNA to target specific components of the calcium signalling pathway may reveal the role of individual molecules involved in calcium responses. Future work will include the study of individual endothelial mechanoreceptors and their role to distinguish different applied flow patterns and convert them to divergent calcium signalling.

Chapter 4: Activation of β1- integrin by unidirectional flow promotes endothelial responses

4.1 Introduction

Endothelial cells are constantly subjected to shear stress, a weak but significant force generated by blood flow. These cells have the feature to sense mechanical stimuli and convert them into biochemical signals, affecting several cellular functions. The mechanical stimuli can be detected by several endothelial mechanoreceptors, that can activate downstream signalling pathways, leading to regulation of endothelial pathophysiology. These mechanoreceptors can be found in different parts of the endothelial cells, such as cell- to- cell junctions, the cytoplasm and the cell membrane. Known endothelial mechanoreceptors include the glycocalyx, the primary cilia, ion channels, such as TRP channels, piezo 1, P2X4 ATP- operated cation channels, adhesion molecules, such as PECAM- 1, VEGFRs and integrins.

The first chapter of the current project showed that endothelial cells convert specific shear stress patterns into distinct calcium signalling. Recent studies have also shown that frequency, direction and magnitude of flow control endothelial inflammatory responses (Feaver et al., 2013, Wang et al., 2013). However, the specific mechanoreceptors that allow endothelial cells to respond specifically to distinct mechanical forces remain unknown. It is plausible that one mechanoreceptor can sense different flow patterns and activate subsequently distinct downstream signalling pathways. Indeed, the function of PECAM- 1, member of a mechanosensory complex located on the cell- to- cell junctions, depends on its anatomical location. To be more specific, PECAM- 1 at the inner curvature of the aorta, where flow has a low and oscillatory shear stress, was found to promote pro- atherosclerotic phenotype (Harry et al., 2008). On the contrary, PECAM- 1 at the descending aorta, where flow has high and laminar shear stress profile, was found to play an atheroprotective role (Goel et al., 2008).

This evidence suggests that a mechanoreceptor, such as PECAM- 1, can distinguish between different mechanical conditions by triggering divergent downstream signalling. On the other hand, it is plausible that some mechanoreceptors can respond to specific mechanical forces only, activating concrete downstream signalling pathways. The latter explanation highlights the unique structural characteristics of some mechanoreceptors, as their mechanical activation may require specific intramolecular structural rearrangements, which can be triggered by unique mechanical conditions.

In order to characterize mechanoreceptors that detect and respond to distinct flow patterns in endothelial cells, calcium dynamics in endothelial cells under flow were assessed after inhibition or stimulation of specific mechanoreceptors.

Hypothesis

A single mechanoreceptor on the endothelial cell surface can be activated and respond to distinct applied flow patterns.

Aims

1. Assess gene expression levels of mechanoreceptors in endothelial cells
2. Test whether β1- integrin is expressed on the apical surface of the endothelium
3. Determine whether β1- integrin can be activated by distinct flow patterns
4. Elucidate if inhibition or stimulation of β1- integrin affects calcium responses in endothelial cells under different flow patterns
5. Identify the molecular pathway involved in β1- integrin mediated calcium responses in endothelial cells under flow
6. Evaluate the long- term flow responses of endothelial cells after inhibition of β1- integrin

4.2 Results

4.2.1 β1- integrin is highly expressed in endothelial cells

Endothelial cells use specific mechanoreceptors in order to sense flow and convert the mechanical stimuli into specific biochemical signals, such as calcium. It was previously shown that endothelial cells mount calcium signalling in response to distinct flow patterns. First of all, a literature review was performed to identify known mechanoreceptors in endothelial cells associated with calcium signalling (Table 4.1). These studies used different techniques to apply mechanical forces on endothelial cells and test their responses. As endothelial cells have a range of mechanoreceptors, located on different cellular compartments, a gene expression analysis was performed in order to examine the expression levels of mechanoreceptors known to play a role in calcium signalling in endothelial cells.

Using RNA isolated from static HUVECs, the assessment of gene expression levels by qPCR revealed that distinct mechanoreceptors are expressed in different levels (Figure 4.1). To be more specific, β1- integrin (itgb1) showed the highest expression levels compared to all other mechanoreceptors. In addition, piezo 1 and trpp2 calcium channels are also highly expressed compared to other mechanoreceptors, such as trpp1 and trpv4. Furthermore, trpp1, trpm4, trpv2 and trpv4, all members of the trpp family of mechanosensitive calcium channels, showed similar gene expression levels. On the contrary, trpc1, also member of the trpp family demonstrated very low expression levels. Finally, p2x4 and p2x7, members of the ATP-gated P2X receptor cation channel family, showed the lowest expression levels compared to all other tested genes.

These data indicate that mechanoreceptors of the endothelial cells are expressed in different levels, with β1- integrin exhibiting the highest expression.

Table 4.1. Known mechanoreceptors of endothelial cells. All these molecules have been previously associated with Ca^{2+} signaling and tested for their contribution to mechanoresponses using different techniques

Name of mechanoreceptor	Technique	Refs.
P2X4	Flow system (ATP needed)	(Yamamoto et al., 2000)
P2X7	Flow system	(Ramirez and Kunze, 2002)
β1- integrin	Flow system, Electromagnetic needle-magnetic beads	(Matthews et al., 2010)
Piezo 1	Patch clamp- Piezo-electrically driven glass probe	(Ranade et al., 2014, Hyman et al., 2017)
TRPP1 (Polycystin 1)	Flow system, knock out mice	(Nauli et al., 2003, Yao and Garland, 2005, Patel, 2015)
TRPP2 (Polycystin 2)	Flow system, blocking antibodies	(Nauli et al., 2003, Yao and Garland, 2005, Patel, 2015)
TRPC1	siRNA, patch clamp	(Chen and Barritt, 2003, Maroto et al., 2005)
TRPM4	Negative pressure-membrane stretch	(Yao and Garland, 2005, Morita et al., 2007)
TRPV2	Membrane stretch	(Muraki et al., 2003, Yao and Garland, 2005)
TRPV4	Flow system, siRNA	(Yao and Garland, 2005, Wu et al., 2007)

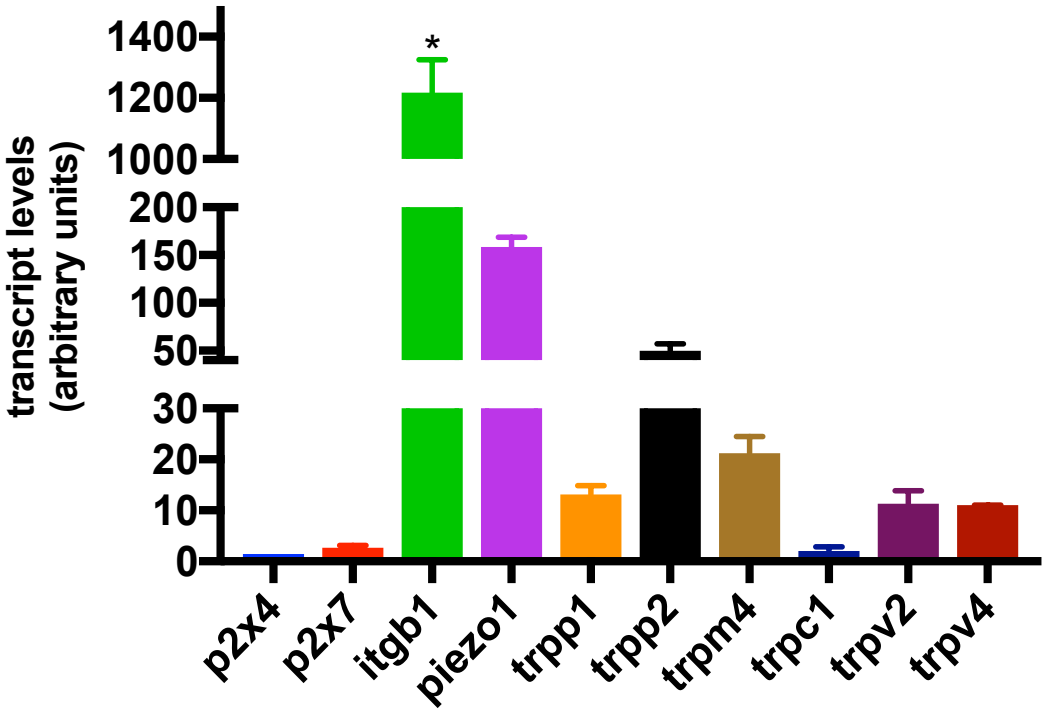


Figure 4.1 Gene expression levels of mechanoreceptors in HUVECs

Total RNA from HUVECs under static conditions was isolated and qRT- PCR was performed to assess the expression levels for multiple mechanoreceptors. Fold differences were calculated using the comparative ΔΔCt method. β1-integrin showed the highest gene expression levels compared to all other mechanoreceptors. All data were normalized to the expression levels of p2x4. (n=3, * indicates p=< 0.05, using a one- way ANOVA test, with Tukey's test for multiple comparisons. Values are ± SEM).

4.2.2 β1- integrin is expressed on both the basal and the apical surface of the endothelial cells

Gene expression analysis revealed that β1- integrin is highly expressed in static HUVECs (Figure 4.1). β1- integrin is a known transmembrane protein facilitating the cell adhesion to the extracellular matrix (ECM). Several studies have highlighted the important role of β1- integrin in cell- ECM interaction and how this interaction can activate integrin to trigger downstream cellular responses (Barczyk et al., 2010, Ross et al., 2013). Therefore, the main pool of β1- integrin is located on the basal surface of the endothelial cells, in close proximity with the ECM.

However, as fluid shear stress is applied directly on the apical surface of the endothelial cells, in order to examine the presence of β1- integrin not only on basal but also on the apical surface, segments from mouse descending aortas were stained *en face* with antibodies targeting either the active or the total β1- integrin (Figure 4.2). 3D reconstruction of images obtained by a super- resolution fluorescent microscope confirmed that β1- integrin is mainly expressed on the basal surface of the endothelial cells but a proportion is expressed on the apical surface (Figure 4.3). Furthermore, it was revealed that active β1- integrin molecules are present on the apical surface of the cells.

These 3D reconstructions confirm that β1- integrin is present on both the basal and the apical surface of endothelial cells.

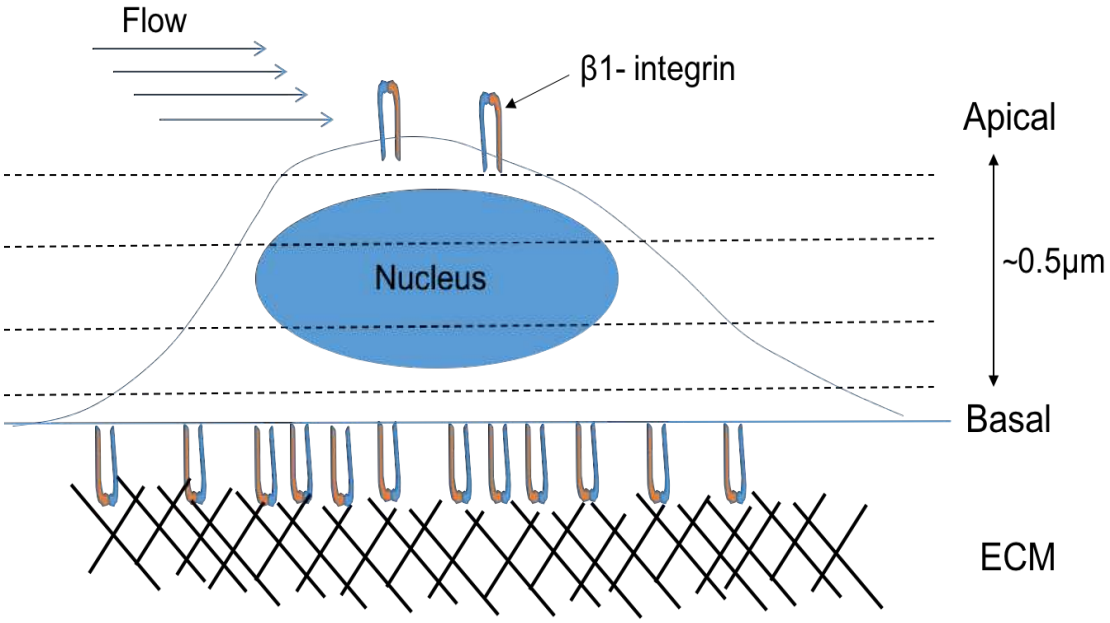
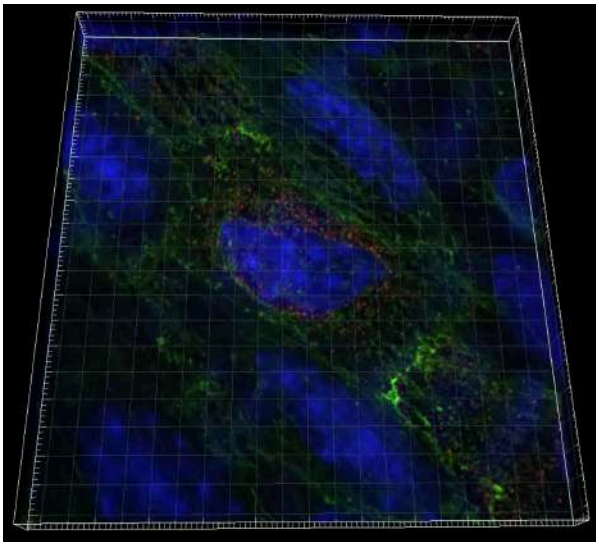
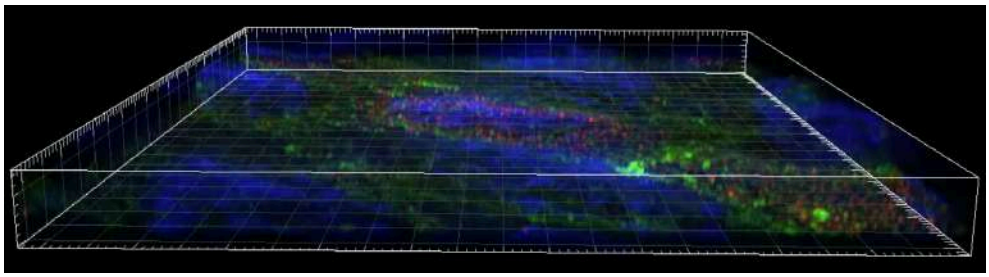


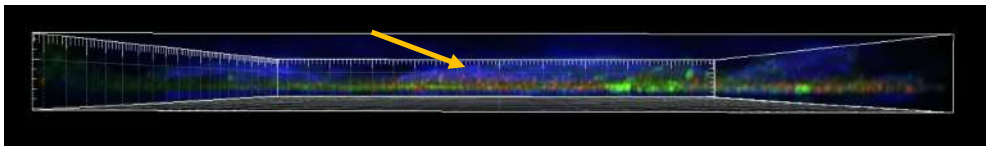
Figure 4.2 Schematic representation showing the location of β1- integrin on endothelial cells

The main pool of β1- integrin is based on the basal surface of the endothelial cells, acting as a "bridge" between the cell and the extracellular matrix (ECM), providing information to the cell about the local ECM environment. As flow is applied on the apical surface of the endothelial cells, it was examined whether β1- integrin is also present on the apical surface.

A







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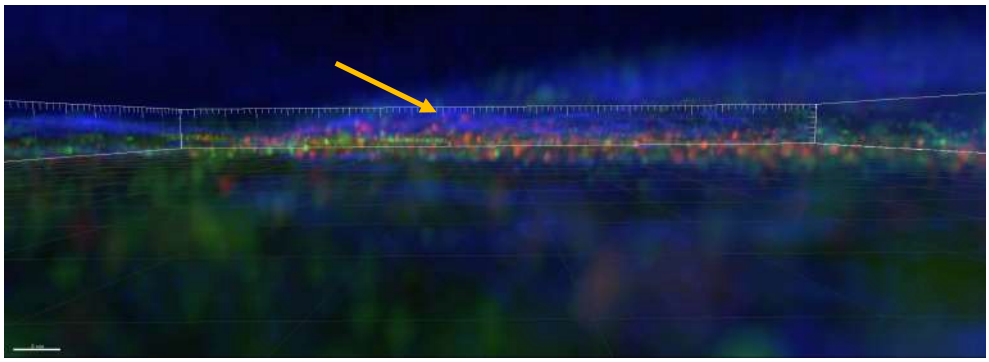

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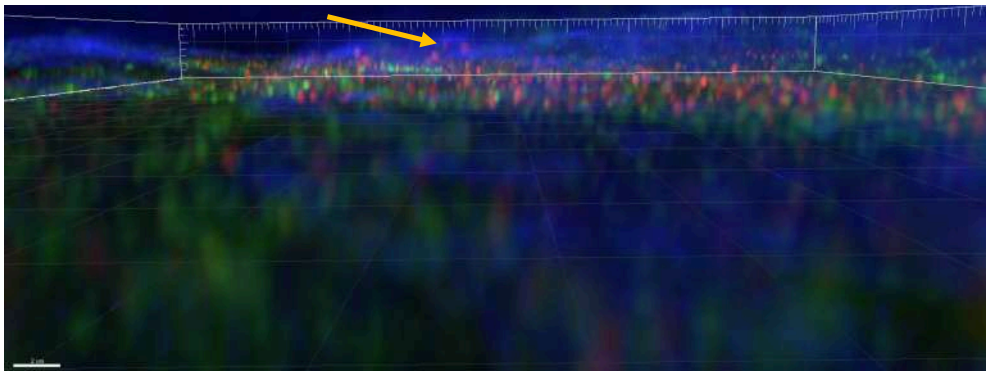

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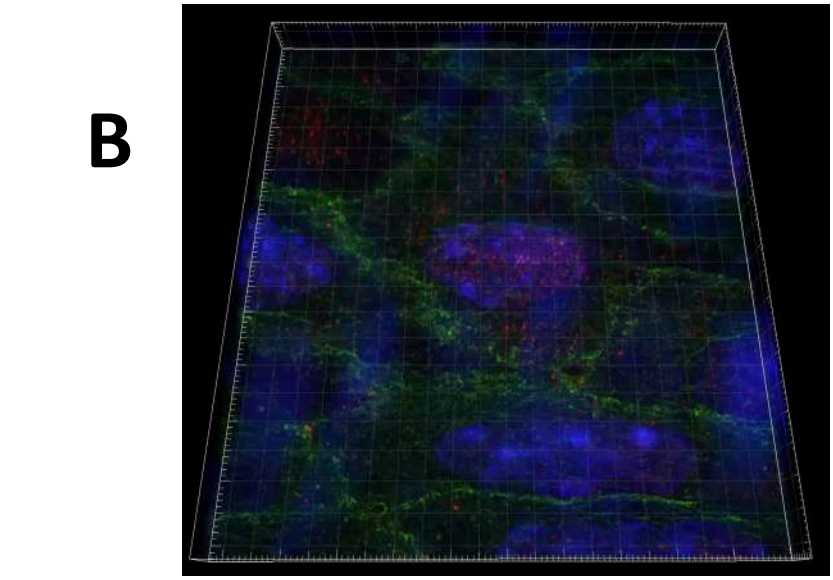

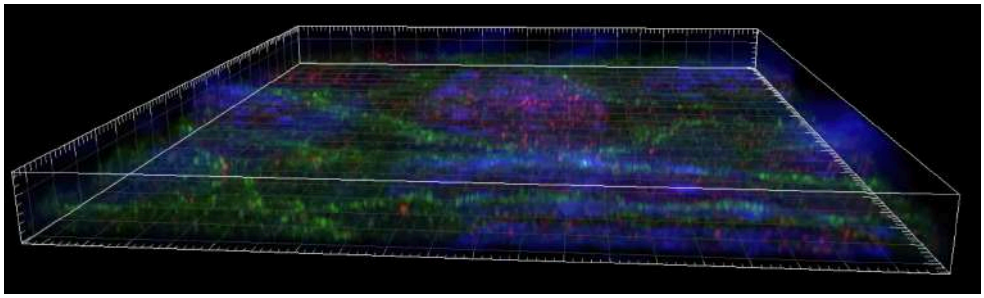

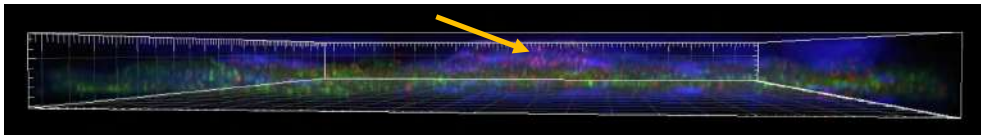

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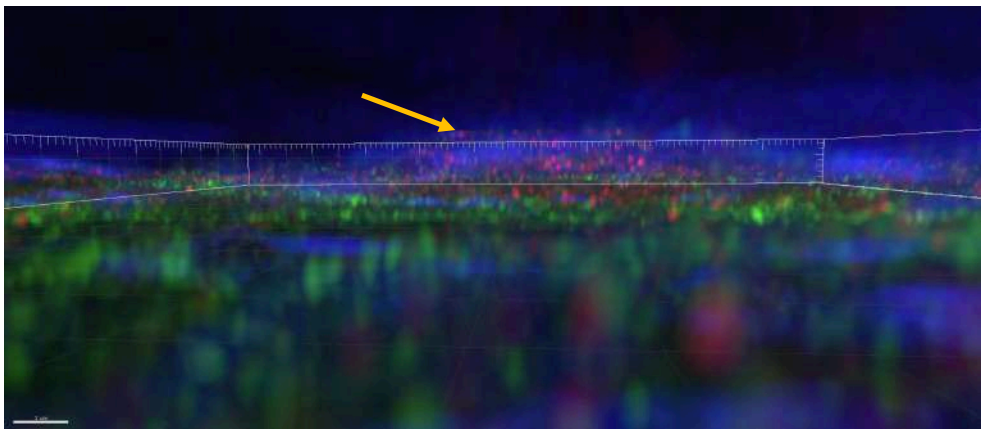

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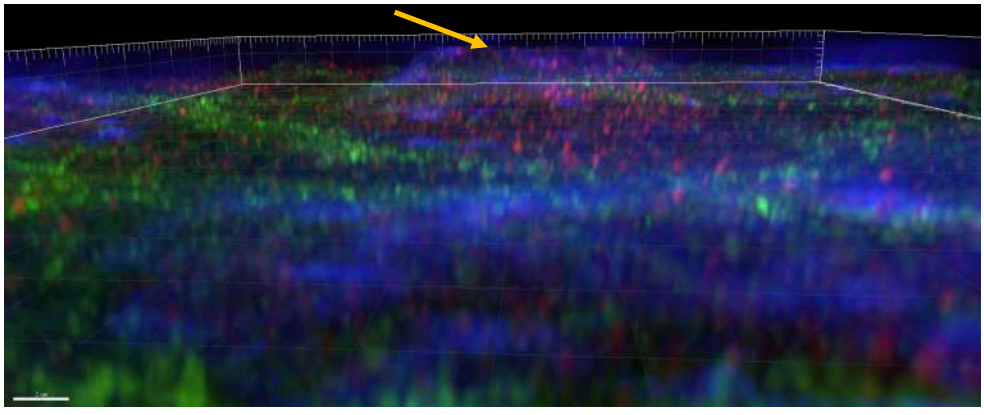

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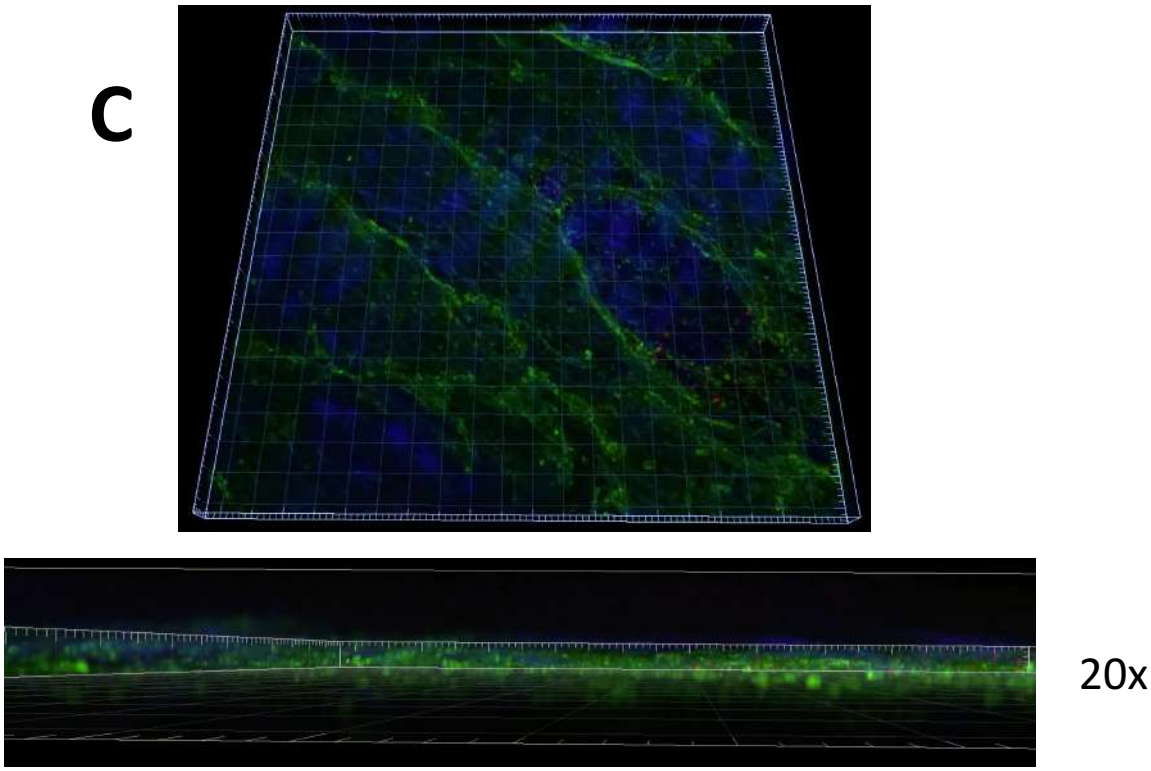


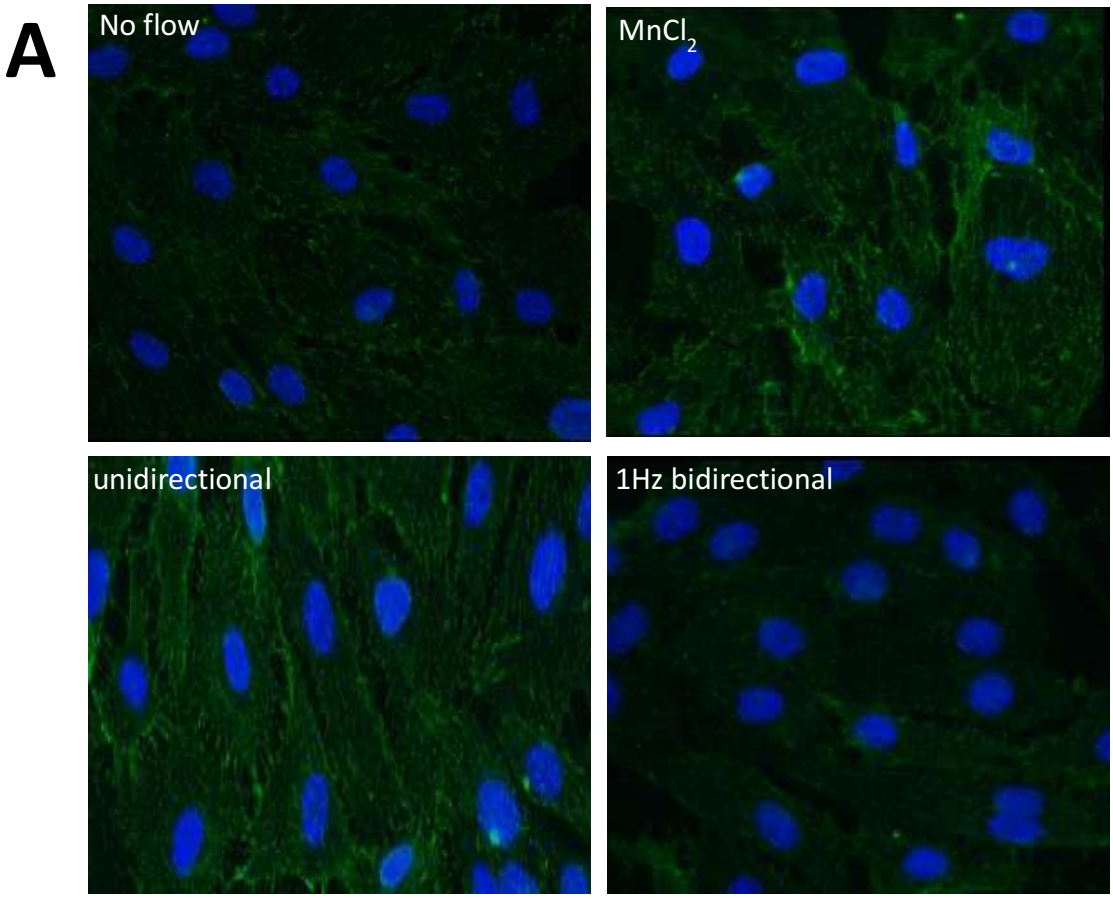
Figure 4.3 β1- integrin is expressed on both the basal and the apical surface of the endothelial cells

Segments of mouse descending aortas were *en face* stained with mab 1997 (red signal) targeting the total β1- integrin (A) or with 9EG7 (red signal) targeting the active β1- integrin (B) or with IgG control (C). The tissue was also stained with a 488- conjugated- CD31 antibody (green signal- endothelial specific marker). To visualise the nucleus, DAPI nuclear staining was used (blue signal).
A super- resolution fluorescent microscope was used to obtain slices of mouse descending aortas at a step of 0.25 μm and a 60x magnification. Then, the stack of images was used to generate 3D reconstructions of the tissue. Different angles of the 3D reconstructions show the presence of either total (A) or active (B) β1- integrin on both the basal and the apical surface at three different magnifications. Apical β1- integrin is marked using yellow arrows.

4.2.3 Unidirectional but not 1 Hz bidirectional flow activates β1-integrin in endothelial cells

β1- integrin is known to have two structural forms depending on its activation state, a bent- inactive state and an extended- active state. β1-integrin activation can be triggered by either intracellular signals (inside-out signalling) or extracellular stimuli (outside- in signalling). Our aim was to test whether β1- integrin can be activated by flow and, more specifically, if the application of different flow patterns has any distinct effect on activation status of the molecule. Therefore, HUVECs were exposed to unidirectional or 1 Hz bidirectional flow of 15 dynes/cm^2 for 3 minutes and then stained with an antibody detecting the active form of β1- integrin only (Figure 4.4 A). As controls, static HUVECs and static HUVECs pre- treated with $MnCl_2$, a known activator of integrins, were also stained with the same antibody. First of all, quantification of the mean fluorescence intensity confirmed that $MnCl_2$ activates β1- integrin, as mean fluorescence was almost 40% increased compared to the untreated static HUVECs (Figure 4.4 B). In addition, application of unidirectional flow caused a significant increase in the fluorescent signal compared to the no flow condition. However, application of 1 Hz bidirectional flow failed to induce a significant increase in the mean fluorescent intensity. Finally, there was no significant difference between cells treated with $MnCl_2$ and cells exposed to unidirectional flow. On the contrary, the mean fluorescence of HUVECs treated with $MnCl_2$ showed a significant increase compared to cells exposed to 1 Hz bidirectional flow.

These data indicate that β1- integrin is activated by unidirectional but not 1 Hz bidirectional flow.


A
No flow
MnCl2
unidirectional
1Hz bidirectional

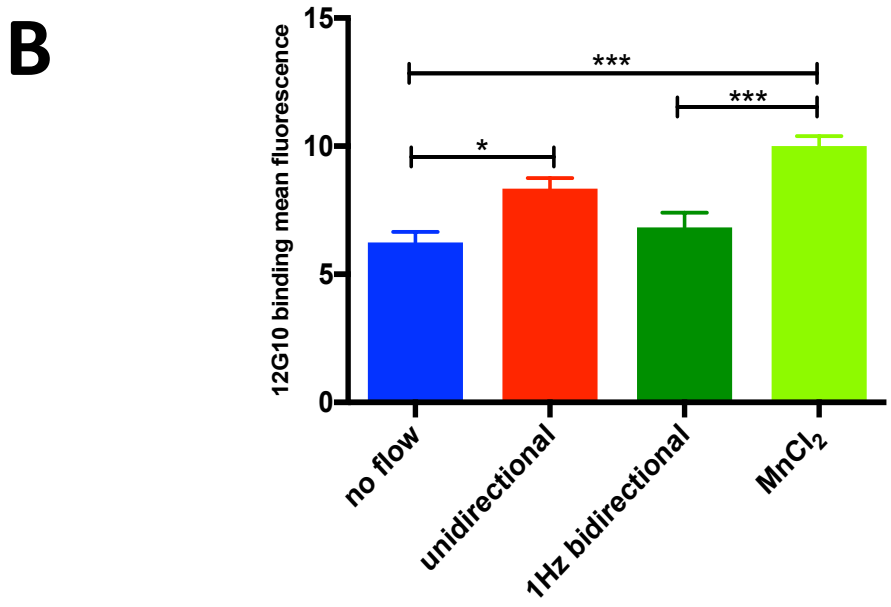

B
12G10 binding mean fluorescence
15
10
5
0
***
***
*
no flow
unidirectional
1Hz bidirectional
MnCl2

Figure 4.4 β1- integrin was activated by unidirectional but not 1 Hz bidirectional flow

(A) Representative images showing active β1- integrin in HUVECs. HUVECs were exposed to unidirectional or 1 Hz bidirectional flow of 15 dynes/cm^2 for 3 minutes and then immediately fixed. Cells were stained with 12G10 antibody (green signal), targeting the active β1- integrin only, and DAPI (blue signal) in order to visualise the nucleus. Static HUVECs and static HUVECs pre- treated with 10 μM of $MnCl_2$ for 30 minutes (known activator of integrins) were also stained with 12G10 and DAPI. Fluorescent images were obtained using 40x magnification of a fluorescent microscope.

(B) Quantification of mean fluorescent intensity. 12G10 binding mean fluorescence of static HUVECs (blue), HUVECs exposed to unidirectional flow (red), HUVECs exposed to 1 Hz bidirectional flow (green) and static HUVECs treated with $MnCl_2$ (light green). 10 random cells were selected in each image using the ImageJ software and then the mean fluorescent intensity of the green signal, indicating the 12G10 binding, was calculated. 2 images of each condition for every experimental repeat were analysed and then the average mean fluorescent intensity was calculated. Exposure of cells to unidirectional and not 1 Hz bidirectional flow increased 12G10 binding mean fluorescence compared to no flow condition indicating the activation of β1- integrin by this specific flow pattern.
(n=5, * indicates p=<0.05, *** indicates p=<0.001 using a one- way ANOVA, with Tukey's test for multiple comparisons. Values are ± SEM)

4.2.4 Inhibition of β1- integrin decreases calcium responses in endothelial cells under unidirectional but not 1 Hz bidirectional flow

Since unidirectional flow can activate β1- integrin, we wished to know if this event was important for calcium signalling in response to shear stress. This was achieved using a blocking antibody (P5D2) that inhibits β1- integrin activation.

First of all, to confirm that P5D2 antibody inhibits activation of β1- integrin, a cell adhesion assay was performed as a control. HUVECs were treated with P5D2 for 45 minutes and then seeded on fibronectin coated dishes (Figure 4.5 A). As controls, HUVECs were treated with IgG control or treated with 12G10 stimulatory antibodies, which are known to activate β1- integrin. Quantification of the number of cells adhered on fibronectin confirmed that P5D2 reduces β1- integrin activation, as the number of adhered cells was 40% decreased compared to IgG treated cells. In addition, the number of adhered cells treated with 12G10 was 40% increased compared to IgG treated cells, confirming that this antibody activates β1- integrin (Figure 4.5 B).

I then examined the effects of P5D2 on calcium signalling under flow. HUVECs loaded with a calcium fluorescent dye, were treated with P5D2 antibodies for 45 minutes and then exposed to unidirectional or 1 Hz bidirectional flow of 15 dynes/cm^2 for 3 minutes. HUVECs treated with an IgG control and exposed to the same flow conditions were used as control. The quantification of the amplitude of the calcium peak showed that HUVECs treated with the inhibitory antibody had 50% decreased calcium responses to unidirectional flow, compared to the IgG control indicating that β1- integrin is involved in the response to unidirectional flow (Figure 4.6 A). However, the calcium response of HUVECs under 1 Hz bidirectional flow was similar in P5D2 and control IgG- treated cells, indicating that β1- integrin is not involved in the response to bidirectional flow.

Finally, to confirm that the addition of the P5D2 inhibitory antibody does not affect the baseline calcium responses in HUVECs, static HUVECs loaded with a calcium fluorescent dye, were treated with IgG control or P5D2 antibody. The measurement of calcium responses showed that although addition of P5D2 inhibitory antibody increased calcium signalling compared to IgG under static conditions, there was no significant difference (Figure 4.6 B).

Overall, these data demonstrate that inhibition of β1- integrin reduces calcium responses in endothelial cells under unidirectional but not under 1 Hz bidirectional flow.

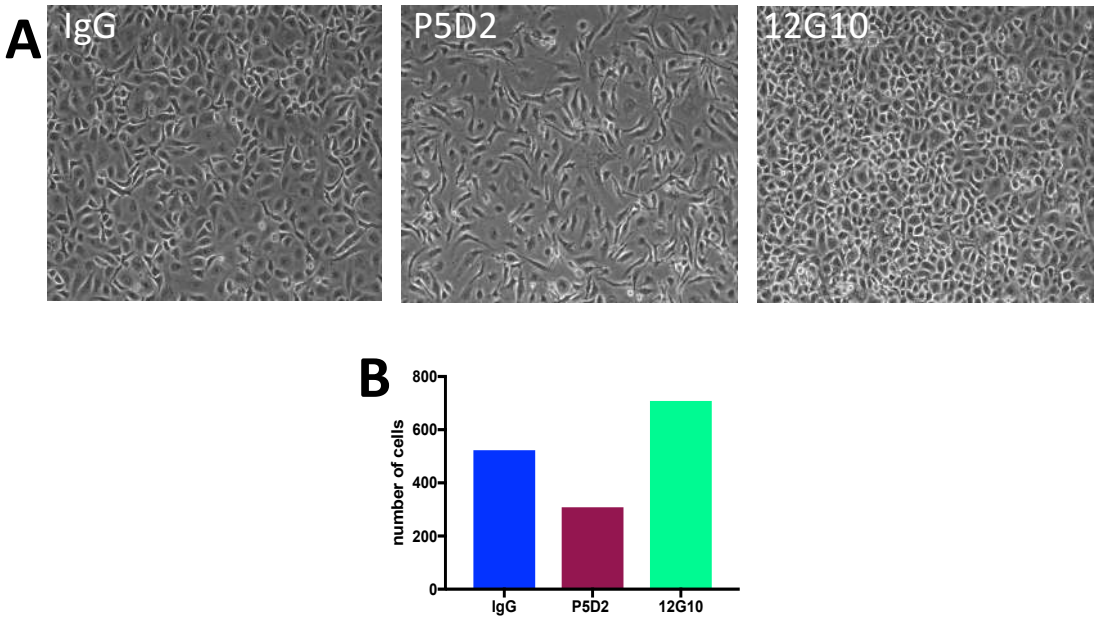


Figure 4.5 P5D2 inhibitory antibody blocks adhesion of HUVECs on fibronectin

HUVECs were treated with 10 µg/ml of IgG, P5D2 inhibitory (blocks activation of β1- integrin) or 12G10 stimulatory (induces activation of β1- integrin) antibody or control IgG for 45 minutes. Cells were then allowed to adhere on fibronectin-coated dishes for 60 minutes. Non- adherent cells were removed by washing. (A) Representative images are shown.

(B) Quantification of number of adhered cells in HUVECs treated with IgG (blue), with P5D2 (maroon red) and with 12G10 (light green). Treatment of cells with P5D2 inhibitory antibody reduced cell adhesion, whereas treatment with 12G10 stimulatory antibody increased the number of adhered cells (n=1).

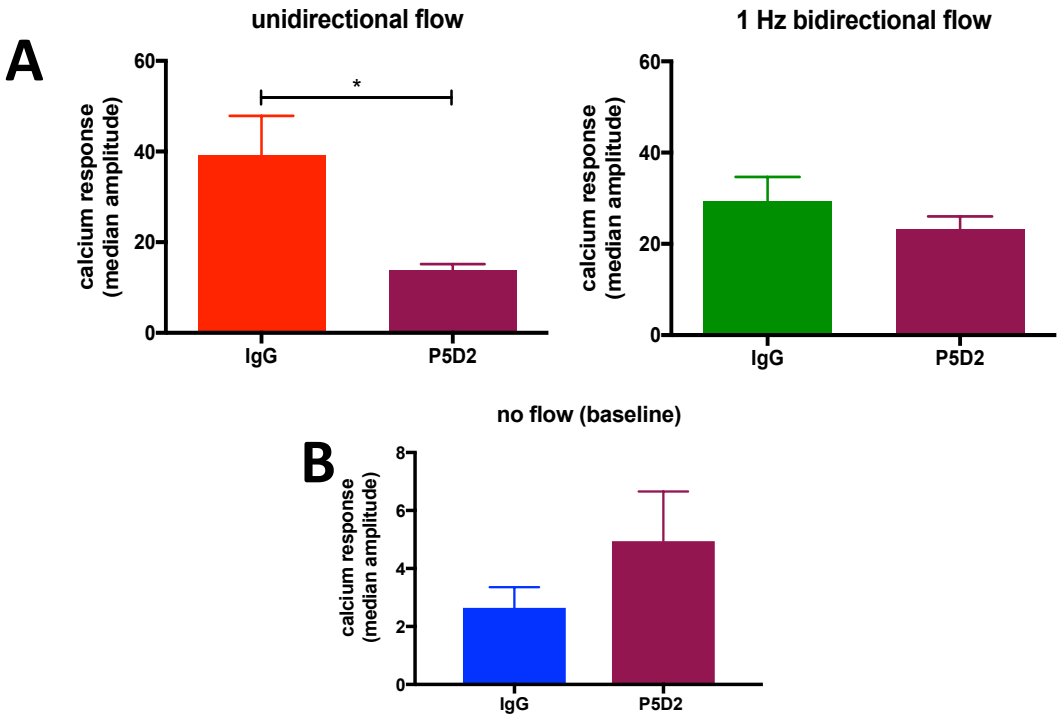


Figure 4.6 Inhibition of β1- integrin reduced calcium signalling in endothelial cells under unidirectional flow only

(A) HUVECs were loaded with calcium fluorescent dye and then treated with 10 µg/ml IgG or P5D2 for 45 minutes. Cells were exposed to either unidirectional (top left graph) or 1 Hz bidirectional flow (top right graph) of 15 dynes/cm^2 for 3 minutes and the calcium responses were recorded using a fluorescent microscope. Treatment of cells with P5D2 inhibitory antibody significantly reduced calcium responses under unidirectional but not 1 Hz bidirectional flow. (n=6. Mean values ± SEM are shown. Statistical analysis was performed using a two- tailed, paired t test* indicates p=< 0.05)

(B) Quantification of calcium responses (median amplitude) in static HUVECs pre- treated with IgG or P5D2 inhibitory antibody. HUVECs were first loaded with calcium fluorescent dye and then treated with IgG or P5D2 for 45 minutes. The calcium responses under static conditions were recorded using a fluorescent microscope. Treatment of cells with P5D2 inhibitory antibody did not significantly affect baseline calcium responses of endothelial cells.
(n=4. Mean values ± SEM are shown and statistical analysis was performed using a two- tailed, paired t test)

4.2.5 Activation of β1- integrin enhances calcium responses in endothelial cells under unidirectional but not 1 Hz bidirectional flow

My next aim was to examine whether the pre- activation of β1- integrin before the application of different flow patterns would affect the calcium responses. In order to test this, HUVECs loaded with a calcium fluorescent dye, were treated with a stimulatory antibody (12G10), which is known to trigger the activation of β1- integrin and to stabilise this form. Then, the cells were exposed to unidirectional or 1 Hz bidirectional flow of 15 dynes/cm^2 for 3 minutes. HUVECs treated with IgG and exposed to the same flow conditions were used as control.

The quantification of calcium dynamics revealed that treatment of HUVECs with 12G10 stimulatory antibody significantly increased calcium responses under unidirectional flow. On the other hand, HUVECs treated with 12G10 stimulatory antibody but exposed to 1 Hz bidirectional flow showed unaltered calcium responses compared to the control (Figure 4.7). Furthermore, to test that the treatment of HUVECs with 12G10 stimulatory antibody does not affect the baseline calcium responses, static HUVECs were loaded with a calcium fluorescent dye and then treated with IgG control or 12G10 antibody. The measurement of calcium responses under static conditions showed no significant differences between the IgG control and cells treated with 12G10 antibody.

These results indicate that pre- activation of β1- integrin increases calcium responses in endothelial cells under unidirectional but not 1 Hz bidirectional flow.

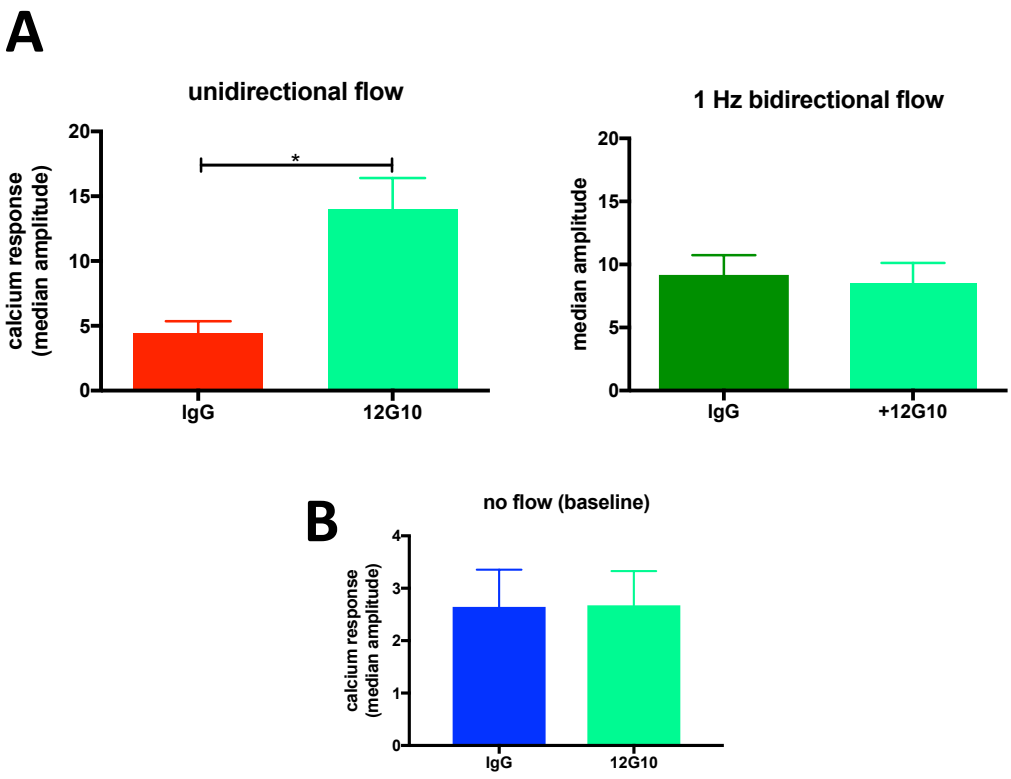


Figure 4.7 Activation of β1- integrin led to enhanced calcium responses in endothelial cells under unidirectional but not 1 Hz bidirectional flow

HUVECs were first loaded with calcium fluorescent dye and then treated with 10 µg/ml IgG or 12G10 for 45 minutes. Cells were exposed to either unidirectional or 1 Hz bidirectional flow of 15 dynes/cm^2 for 3 minutes (A) or remained under static conditions as a control (B). Calcium responses were recorded using a fluorescent microscope. Mean values ± SEM are shown. Differences were tested using a two- tailed, paired t test (n=5, * indicates p=< 0.05). Treatment of cells with 12G10 stimulatory antibody significantly enhanced calcium responses under unidirectional but not 1 Hz bidirectional flow.

4.2.6 Phospholipase C regulates β1- integrin- mediated calcium responses in endothelial cells under unidirectional flow

Inhibition of Phospholipase C in endothelial cells under unidirectional flow significantly reduced calcium responses (Figure 3.6). Therefore, it was examined whether Phospholipase C also regulates β1- integrin mediated calcium responses in endothelial cells under unidirectional flow. To test this, HUVECs loaded with a calcium fluorescent dye were treated with 12G10 stimulatory antibody or with IgG control. Then, cells were treated with the phospholipase C inhibitor (U- 73122) or were left untreated and then exposed to unidirectional flow for 3 minutes.

Analysis of the calcium peak amplitude showed that HUVECs prior treated with 12G10 stimulatory antibody showed a significant increase in calcium responses compared to control IgG- treated cells but this difference was abolished in cells pre- treated with U- 73122 (Figure 4.8)

These data suggest that Phospholipase C regulates calcium responses downstream from the β1- integrin- mediated pathway in endothelial cells exposed to unidirectional flow.

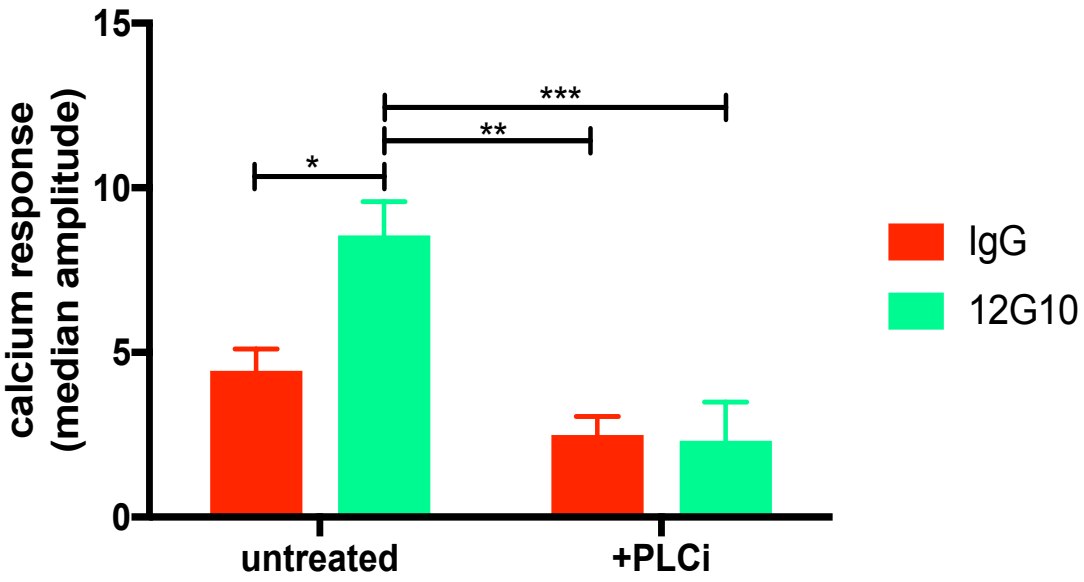


Figure 4.8 Phospholipase C inhibitor blocked β1- integrin mediated calcium responses in endothelial cells under unidirectional flow

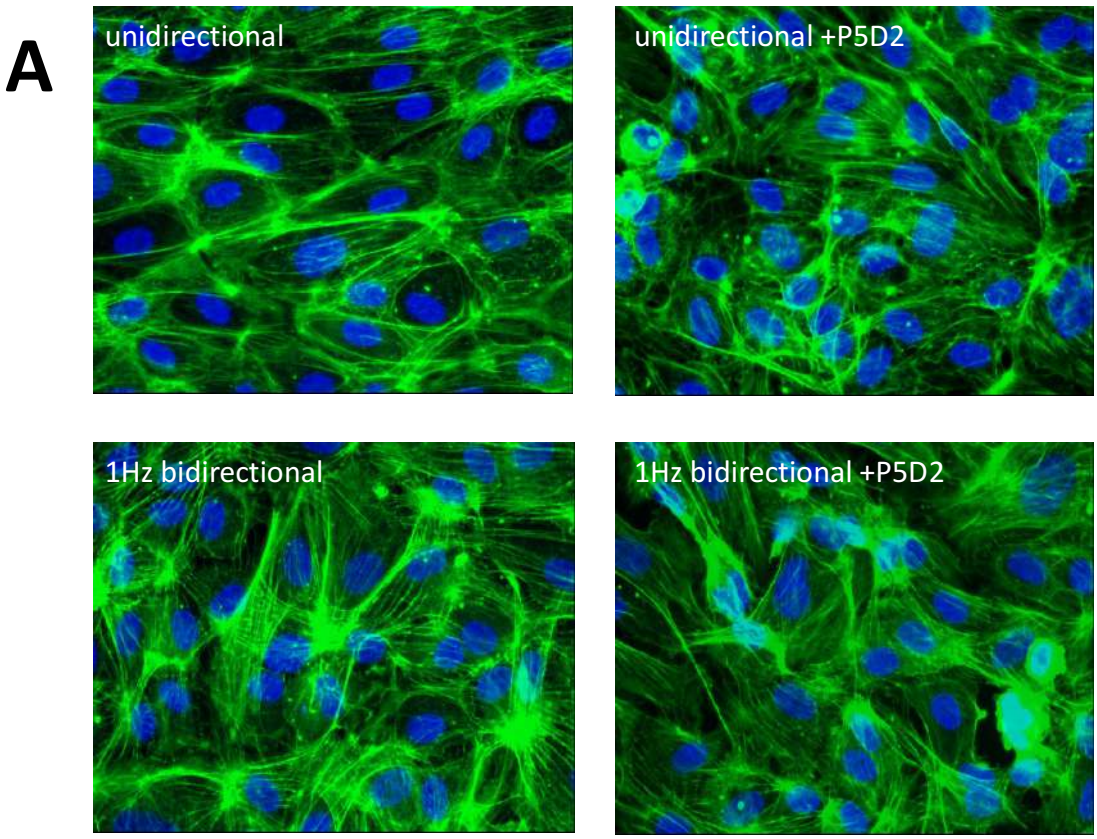
HUVECs were first loaded with calcium fluorescent dye and then treated with 10 µg/ml IgG or 12G10 for 45 minutes. Then cells were treated with phospholipase C inhibitor (PLCi: U- 73122) for 15 minutes or left untreated. Cells were exposed to unidirectional flow of 15 dynes/cm^2 for 3 minutes and the calcium responses were recorded using a fluorescent microscope. Mean values ± SEM are shown and statistical analysis was performed using a 2- way ANOVA test (n=5, * indicates p=< 0.05, ** indicates p=< 0.01, *** indicates p=< 0.001). Treatment of cells with PLCi abolished the enhancement of calcium responses under unidirectional flow in HUVECs pre- treated with 12G10 stimulatory antibody.

4.2.7 Inhibition of β1- integrin disorganises the actin cytoskeleton of endothelial cells under unidirectional flow

Our next aim was to test whether inhibition of β1- integrin would affect cellular responses under prolonged application of flow. Studies have shown that prolonged application of unidirectional flow causes alignment of the cell body to the direction of unidirectional flow (Steward et al., 2015). Therefore, we tested the alignment of the actin cytoskeleton in endothelial cells under unidirectional or 1 Hz bidirectional flow of 15 dynes/cm^2 for 24 hours, with or without inhibition of β1- integrin.

HUVECs were treated with P5D2 inhibitory antibody or IgG control and exposed to the above flow patterns. After 24 hours, cells were fixed and stained with an actin cytoskeleton marker in order to visualise the actin fibers (Figure 4.9 A). Analysis of the alignment of the actin fibers showed that cells exposed to unidirectional flow of high shear stress had significantly more aligned actin fibers compared to cells exposed to 1 Hz bidirectional flow. Furthermore, the pre- treatment with P5D2 abolished the actin fiber alignment in cells exposed to unidirectional flow (Figure 4.9 B).

These results indicate that inhibition of β1- integrin blocks the alignment of the actin cytoskeleton in endothelial cells under unidirectional flow.


A
unidirectional
unidirectional +P5D2
1Hz bidirectional
1Hz bidirectional +P5D2

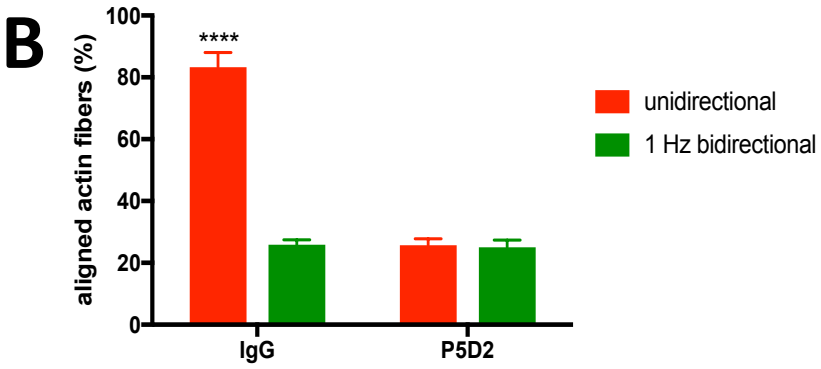

B
aligned actin fibers (%)
100
80
60
40
20
0
****
IgG
P5D2
unidirectional
1 Hz bidirectional

Figure 4.9 Inhibition of β1- integrin led to misalignment of the actin cytoskeleton under unidirectional flow

HUVECs pre- treated under static conditions with 10 μg/ml IgG control or P5D2 inhibitory antibody and then exposed to unidirectional or 1 Hz bidirectional flow of 15 dynes/cm^2 for 24 hours. The circulating fluid contained 1 μg/ml IgG control or P5D2 inhibitory antibody. After 24 hours, cells were fixed and stained with phalloidin (green) in order to visualise the actin fibers or DAPI (blue) to visualise the nucleus. Fluorescent images were obtained using 40x magnification of a fluorescent microscope and representative images are shown in (A).

Using ImageJ software, 20 random cells were selected in each image and alignment of actin fibers was quantified (B). Actin fibers were considered "aligned" if their direction was parallel to the direction of the cell major axis (within 30° of the major axis) and "misaligned" if not (Baeyens et al., 2014). 2 images of each condition for every experimental repeat were analysed and then the average percentage of aligned actin fibers was calculated. Mean values ± SEM are shown and statistical analysis was performed using a 2- way ANOVA test (n=3, **** indicates p=< 0.0001). Treatment of HUVECs with P5D2 inhibitory antibody blocked the alignment of actin fibers under unidirectional flow.

4.3 Discussion

4.3.1 Conclusions

- β1- integrin is highly expressed in endothelial cells
- β1- integrin is present on both the basal and the apical surface of the endothelial cells
- β1- integrin is activated by unidirectional but not 1 Hz bidirectional flow
- Calcium responses in endothelial cells under unidirectional but not under 1 Hz bidirectional flow are positively regulated by β1- integrin
- Activation of β1- integrin leads to increased calcium signalling in endothelial cells exposed to unidirectional but not 1 Hz bidirectional flow
- Phospholipase C regulates β1- integrin- mediated calcium responses in endothelial cells under unidirectional flow
- Inhibition of β1- integrin leads to misalignment of the actin cytoskeleton in endothelial cells under unidirectional flow

4.3.2 Discussion and Future work

β1- integrin expression

This study has shown that a single mechanoreceptor (β1- integrin) responds to a specific flow pattern (unidirectional flow), by inducing calcium responses in HUVECs. β1- integrin was highly expressed in HUVECs compared to all other mechanoreceptors tested. However, the expression levels of these mechanoreceptors were assessed in static HUVECs. The gene expression levels of mechanoreceptors in static HUVECs may differ from the levels of expression in HUVECs exposed to flow for several hours.

Studies have shown that different flow patterns can affect the gene expression levels of endothelial mechanoreceptors (Wasserman et al., 2002, Chatzizisis et al., 2007). In addition, the expression of mechanosensitive genes may depend on the anatomical position of the endothelial cells and the local flow dynamics, which may be related to the progression of the atherosclerotic plaque. To be more specific, a study from Di Taranto showed that the expression levels of β1- integrin is significantly decreased in endothelial cells of an atherosclerotic plaque compared to a control artery (Di Taranto et al., 2012). Further studies could include gene expression analysis of various endothelial mechanoreceptors under prolonged application of flow.

Apical β1- integrin as a mechanoreceptor

As fluid shear stress is applied on the apical surface of the endothelial cells, a shear stress mechanoreceptor may be located on this surface in order to sense directly the applied flow. Several studies have shown the presence of mechanoreceptors, such as piezo 1 or the primary cilia, on the apical surface of the endothelial cells (Coste et al., 2010, Egorova et al., 2012). Furthermore, a number of studies have studied the role of integrins on the basal membrane of endothelial cells and how they transmit mechanical signals from the ECM into the cell (Shyy and Chien, 2002, Silva et al., 2008). Although other studies have already shown the presence of β1- integrin on the apical surface of the endothelial cells (Yang and Rizzo, 2013), we confirmed this result by using a super- resolution fluorescent microscope in order to image *en face* stained mouse descending aortas. Further experiments to confirm this finding could include the use of atomic force microscopy or electro- microscopy.

I used an inhibitory antibody to block the activation of β1- integrin and then test the effect of its inhibition on calcium dynamics under flow. Endothelial cells are tightly connected to each other forming a monolayer.

Therefore, the treatment of cells with an inhibitory antibody for a short time would block the molecules, located on the apical surface only, as the access to the basal surface would be limited. It should be noted that although the treatment of HUVECs with the inhibitory antibody against β1- integrin caused a significant decrease in calcium signalling under unidirectional flow only, it failed to eliminate the calcium responses triggered by flow (figure 4.6; calcium responses in P5D2- treated cells under unidirectional flow were higher than treated cells under no flow). The main reason of this phenomenon can be the involvement of other endothelial mechanoreceptors in calcium responses under flow. Application of unidirectional flow could activate these mechanoreceptors and induce calcium signalling, even if β1- integrin is blocked. However, the fact that inhibition of β1- integrin reduces calcium responses by 50% under unidirectional flow, highlights the role of β1- integrin as an essential endothelial mechanoreceptor. These data suggest that apical β1- integrin is required to sense unidirectional flow.

Further evidence for the role of apical β1- integrin was obtained by applying activating 12G10 antibodies to the endothelial apical surface. Activation of β1- integrin before the application of unidirectional but not 1 Hz bidirectional flow led to enhancement of the calcium responses in endothelial cells. Studies have demonstrated that activation of integrins with either stimulatory antibodies or their ligands, such as fibronectin, collagen etc, triggers the dissociation of the cytoplasmic legs of the molecule and subsequent rearrangement of the cytoskeleton (Tzima et al., 2001, Askari et al., 2009, Campbell and Humphries, 2011). Therefore, the increase in calcium responses under unidirectional flow after pre- activation of β1- integrin could be explained by the altered structure of the cytoskeleton triggered by the dissociation of β1- integrin cytoplasmic legs. Thus, apical β1- integrin is important because the response to shear is modified by antibodies that target this pool of β1- integrin.

However, the basal integrins may also be important for flow sensing. Integrins can function as traction receptors sensing changes in mechanical forces exerted on the extracellular matrix (Ross et al., 2013). Studies have shown that exposure of endothelial cells to flow activates integrins on the basal surface via a PECAM-1- regulated mechanism (Tzima et al., 2001). In addition, mechanical stretch is applied on the basal surface of the endothelial cells. This type of force can also be sensed by integrins based on the basal surface (Katsumi et al., 2004). Although basal β1- integrin has a significant role providing the cells with information about the local mechanical micro- environment, its contribution has not been studied in the current project. It is possible that the mechanical activation of apical β1- integrin leads to activation of the basal pool of β1- integrin as well. Therefore, there may be a crosstalk between apical and basal pools of integrins, with apical β1- integrin sensing unidirectional shear stress directly and basal integrins to function as direct sensors of mechanical stretch and indirect sensors of shear stress.

Studies have shown that Phospholipase C crosstalks with integrins, as integrin activation leads to phosphorylation and activation of Phospholipase C (outside- in signalling), but also Phospholipase C activation can trigger integrins' activation (inside- out signalling) (Elvers et al., 2010, Zarbock et al., 2012). The use of a pharmacological inhibitor of Phospholipase C in endothelial cells previously treated with a β1- integrin stimulatory antibody, significantly decreased calcium responses under unidirectional flow, confirming that Phospholipase C is part of the β1- integrin mediated signalling pathway leading to calcium release in endothelial cells under flow. Further studies could include the use of siRNA against specific isoforms of Phospholipase C in order to determine the specific components of the pathway.

β1- integrin signalling to actin cytoskeleton

The activation of β1- integrin under unidirectional flow affects not only acute responses to flow, such as calcium dynamics, but also more extensive and chronic cellular responses, such as cytoskeletal rearrangements. The inhibition of β1- integrin led to impaired alignment of the actin cytoskeleton under unidirectional flow, a well- documented endothelial response to unidirectional flow (Van der Meer et al., 2010, Dolan et al., 2011). Other studies have also shown that application of bidirectional flow with low shear leads to misalignment of the endothelial cytoskeleton (Potter et al., 2011). Furthermore, the experiment of the current project testing actin cytoskeletal alignment under different flow patterns, revealed that the direction and not the magnitude of flow is responsible for alignment, as 1 Hz bidirectional flow of high shear stress also failed to induce alignment. As β1- integrin interacts with the actin cytoskeleton via other molecules, such as talin and vinculin, further studies could examine how these components regulate distinct responses in endothelial cells under different flow patterns. The activation of β1- integrin seems to be a crucial point for several cellular responses. Therefore, we tested whether β1- integrin is activated by specific flow patterns and proved that unidirectional flow only can activate the molecule. This is the first study to show that a mechanoreceptor is activated by a specific flow pattern. However, the activation caused by the application of unidirectional flow could be indirect. To be more specific, several mechanoreceptors sense the application of flow on the endothelial cell surface. These mechanoreceptors could trigger the activation of β1- integrin. As a result, although it was shown that β1- integrin is activated by a specific flow pattern, further experiments will be required to prove the direct activation of the molecule by unidirectional and not 1 Hz bidirectional force.

These experiments could include the use of magnetic beads coated with β1- integrin- specific antibodies, which have been previously used to test β1- integrin mechanoresponse (Matthews et al., 2010, Collins et al., 2012).

In summary, β1- integrin is activated and triggers endothelial responses under unidirectional flow only. The next chapter will test the direct mechanical activation of β1- integrin and which specific structural regions of the molecule are responsible for mechanosensation in endothelial cells. These experiments will include the use of an electromagnetic tweezers platform that can generate forces in different directions on beads coated with antibodies targeting different regions of β1- integrin.

Chapter 5: Mechanical activation of β1- integrin by unidirectional force enhances calcium responses in endothelial cells

5.1 Introduction

My data revealed that different flow patterns exert different effects on β1- integrin activation and calcium signalling in endothelial cells. Crystal structures of integrins bound to their ligands have revealed unique conformations before and after integrin activation (Xiong et al., 2001). The heterodimer, consisting of α and β subtypes, can adopt a bent- closed, an extended- closed and an extended- open conformational status (Su et al., 2016, Li et al., 2017). The study of changes in integrin conformational status has been made possible with the use of monoclonal antibodies detecting conformation- specific epitopes of either the inactive or the active form of the molecule (Byron et al., 2009, Mould et al., 2016). Some of the antibodies binding to the high- affinity status will also activate the receptor, whereas others do not exhibit this stimulatory activity and will only bind to the existing active pool of integrins.

The inactive form of integrins is considered to represent the low affinity for the ligand conformation status. Following the activation of the molecule, several intra- module and inter- module movements lead to the unbending of the receptor (Beglova et al., 2002, Liddington and Ginsberg, 2002). Consequently, its activation drives the separation of the transmembrane and cytoplasmic domains, triggering downstream signalling pathways including those driven by build-up of intracellular calcium.

Although activation of β1- integrin due to ligand binding has been previously shown, it is still unknown whether direct application of force can induce intramolecular structural rearrangements leading to its direct activation in a ligand- independent manner. Moreover, it is not clear which specific regions of the molecule are required for mechanosensation. Therefore, it is of great importance to test the direct mechanical activation of β1- integrin and the subsequent downstream effects, such as calcium signalling, in endothelial cells.

Hypothesis

β1- integrin is activated by specific mechanical forces leading to distinct calcium signalling in endothelial cells.

Aims

1. Assess calcium responses in endothelial cells after direct stimulation of β1- integrin with distinct mechanical forces
2. Determine the structural region on β1- integrin responsible for mechanosensation
3. Identify the molecular pathway involved in β1- integrin mediated calcium responses due to application of mechanical forces

5.2 Results

5.2.1 Unidirectional but not bidirectional force activates β1- integrin in endothelial cells

It was previously shown that unidirectional flow activates β1- integrin (Figure 4.4). In order to test whether β1- integrin can be activated directly by distinct mechanical forces, unidirectional or 1 Hz bidirectional force was applied via magnetic beads coated with β1- integrin- specific antibodies.

Firstly, the binding properties of magnetic beads coated non-covalently with 4B4 antibodies (targeting the inactive β1- integrin) or with control IgG were assessed by incubating them with HUVECs for 30 minutes. Using a magnetic tweezers platform, a unidirectional force (~16 pN) was then applied onto the beads for 3 minutes. Beads coated with IgG-control did not bind to the endothelial cells and moved in the direction of the magnetic field forming clusters of beads (Figure 5.1 A). On the contrary, beads coated with 4B4 antibodies remained attached on the endothelial surface after 3 minutes of constant application of force (Figure 5.1 B). This suggests that magnetic beads coated with specific antibodies bind to their target steadily.

In order to test mechanical activation of β1- integrin directly, HUVECs were incubated with 4B4- coated magnetic beads and then unidirectional or 1 Hz bidirectional force (~16 pN) was applied for 3 minutes. Cells were then fixed and stained with 9EG7 antibodies, which specifically detects the active form of β1- integrin. Note that the cells were not permeabilised and therefore the antibodies could only recognise the apical active β1- integrin. As a control, 4B4- coated magnetic beads attached on HUVECs remained under no force for 3 minutes, fixed and stained with 9EG7 antibodies (Figure 5.2 A).

It was observed that the application of unidirectional force caused a significant increase in 9EG7 accumulation near to the beads (indicating the presence of active β1- integrin) whereas beads under no force or 1 Hz bidirectional force did not accumulate 9EG7 (Figure 5.2 B). In addition, the application of distinct mechanical forces did not have any notable effects on the arrangement of the actin cytoskeleton.

These data indicate that β1- integrin is activated directly by the application of unidirectional but not 1 Hz bidirectional force.

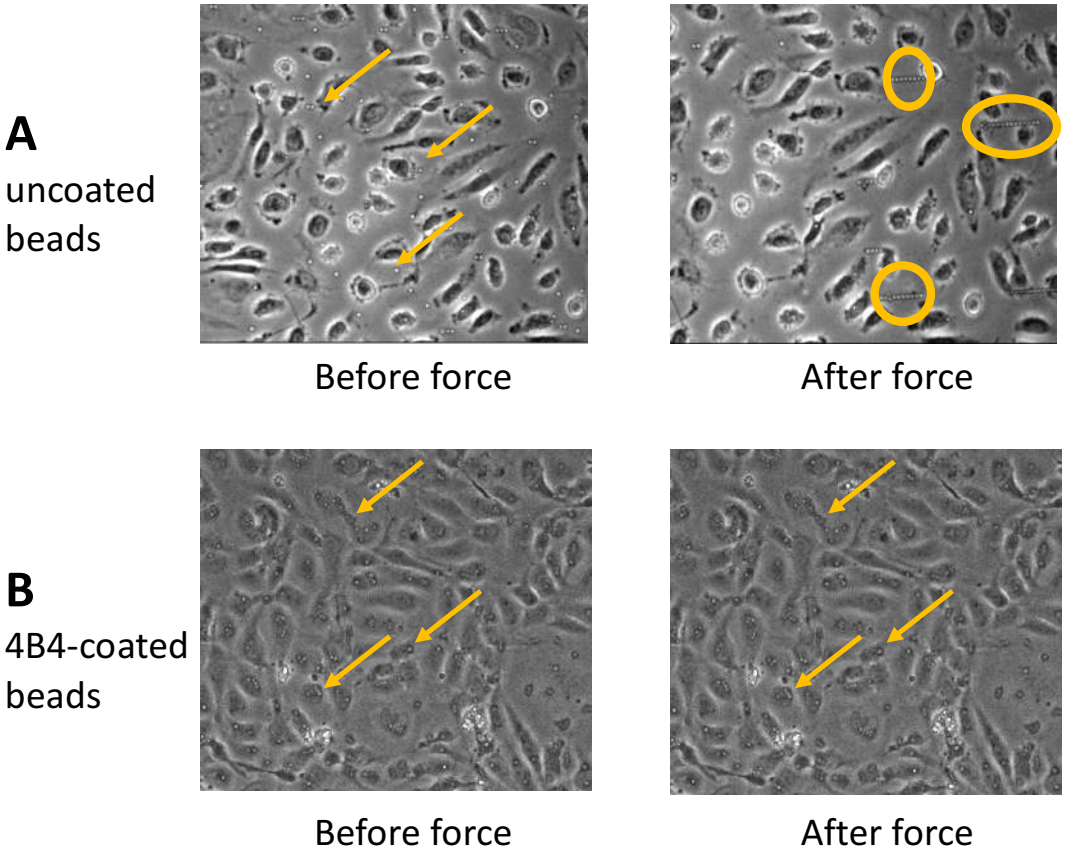


Figure 5.1 Binding of 4B4- coated magnetic beads to β1- integrin in endothelial cells

Magnetic beads were coated non- covalently with IgG control or 4B4 antibodies, targeting the inactive β1- integrin. Beads were incubated with HUVECS for 30 minutes and then exposed to unidirectional force (16 pN) for 3 minutes. Phase-contrast images were taken before and after the application of force. As IgG-control coated beads remained unbound (yellow arrows, top left image), the application of force caused their movement in the direction of the magnetic field and the formation of clusters of beads (yellow circles, top right image). On the contrary, 4B4- coated beads attached on the endothelial cell surface either before (yellow arrows, bottom left image) or after (yellow arrows, bottom right image) the application of force.

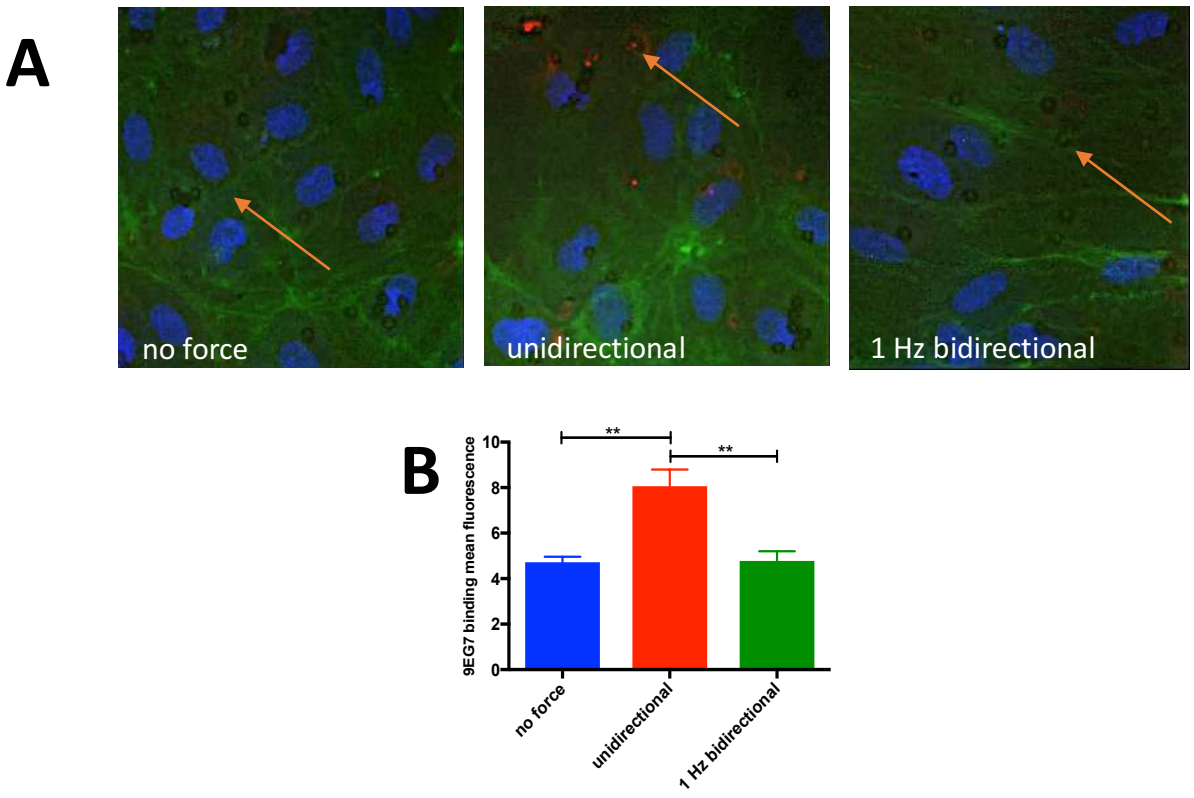


Figure 5.2 Unidirectional and not 1 Hz bidirectional force activated β1-integrin in endothelial cells

(A) Representative images showing active β1- integrin in HUVECs. HUVECs were incubated for 30 minutes with 4B4- coated magnetic beads (targeting the inactive β1- integrin only). Then, beads were exposed to unidirectional or 1 Hz bidirectional force (~16 pN) for 3 minutes. As a control, beads remained under no force for 3 minutes. HUVECs were then fixed and stained with 9EG7 antibodies (red signal), targeting the active β1- integrin only, with phalloidin (green signal) and with DAPI to visualise the nucleus (blue signal). The beads show as grey "dots" (yellow arrows). Fluorescent images were obtained using 40x magnification of a fluorescent microscope. Note the localised red fluorescent signal to unidirectional force only.

(B) Quantification of mean fluorescent intensity of the red signal around the beads, indicating the presence of active β1- integrin. 9EG7 binding was assessed by measuring mean fluorescence of the red signal around the beads exposed to no force (blue), exposed to unidirectional force (red) and to 1 Hz bidirectional force (green). The area around the beads was selected in each image using the ImageJ software and then the mean fluorescent intensity of the Alexa- 568 antibody (red fluorescent signal), indicating the 9EG7 binding, was calculated. 2 images of each condition for every experimental repeat were analysed and then the average mean fluorescent intensity was calculated.
Application of unidirectional force significantly increased the 9EG7 binding mean fluorescence, indicating the direct mechanical activation of β1- integrin by this specific force. (n=4, ** indicates p=<0.01 using a one- way ANOVA, with Tukey's test for multiple comparisons. Values are ± SEM)

5.2.2 Unidirectional and not 1 Hz bidirectional force on β1- integrin induces calcium responses in endothelial cells

In order to investigate whether direct application of distinct forces onto β1- integrin induces calcium signalling in endothelial cells, unidirectional or 1 Hz bidirectional force was applied via magnetic beads coated with antibodies that target β1- integrin (Figure 5.3). In addition, it was tested whether the activation status of β1- integrin plays a role in inducing distinct calcium responses. Thus, forces were applied on beads coated with antibodies targeting either the active or the inactive β1- integrin.

HUVECs were first loaded with a calcium fluorescent dye and then incubated with beads targeting either the active (12G10- coated beads) or the inactive (4B4- coated beads) β1- integrin. As a control, HUVECs were also incubated with beads coated with Poly-D lysine, which binds non-selectively to the plasma membrane. Unidirectional or 1 Hz bidirectional force (~16 pN) was applied onto the beads for 3 minutes and calcium responses were recorded using a fluorescent microscope. As a control, calcium responses of HUVECs incubated with beads but under no force were recorded for 3 minutes.

The quantification of the calcium dynamics in responding cells revealed that application of unidirectional force on beads, targeting either the active or the inactive β1- integrin, induced a significant increase in calcium responses compared to the cells under no force (Figure 5.4). On the contrary, application of 1 Hz bidirectional force failed to trigger a significant calcium response. In addition, application of forces on beads coated with Poly- D lysine did not have a significant effect on calcium signalling, indicating the specificity of the signal coming from 12G10- or 4B4- coated beads under unidirectional force.

These data suggest that both the active and inactive β1- integrin responds to unidirectional but not to bidirectional force.

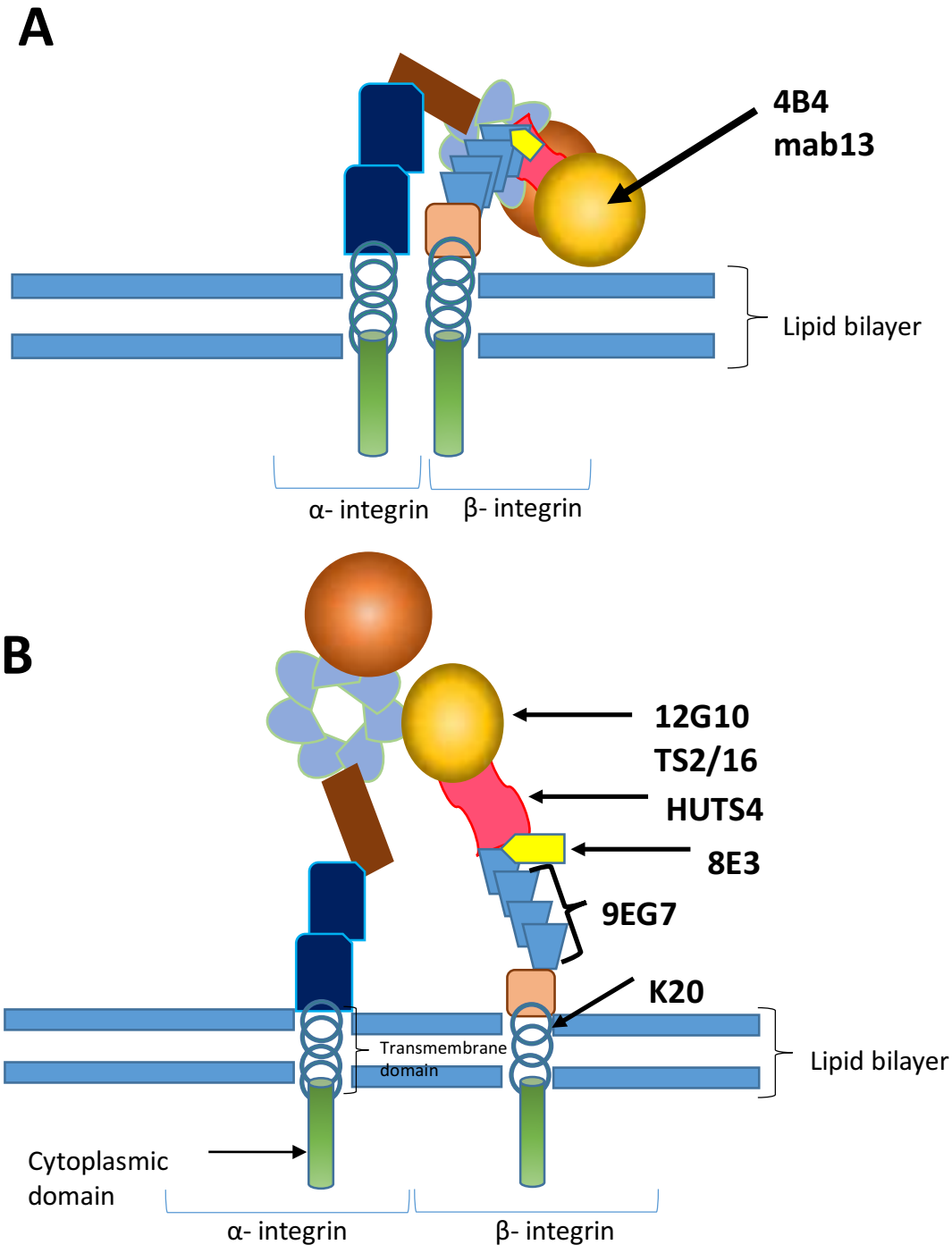


Figure 5.3 Schematic representation of binding sites of different antibodies on the inactive (A) or the active (B) β1- integrin.

4B4 and mab13 bind to the head region of the inactive β1- integrin. 12G10 and TS2/16 bind to the head region of the active β1- integrin. In the active form of β1- integrin, HUTS4 binds to the hybrid domain, 8E3 binds to the PSI domain, 9EG7 binds to the EGF repeats and K20 binds to the close to the membrane region.

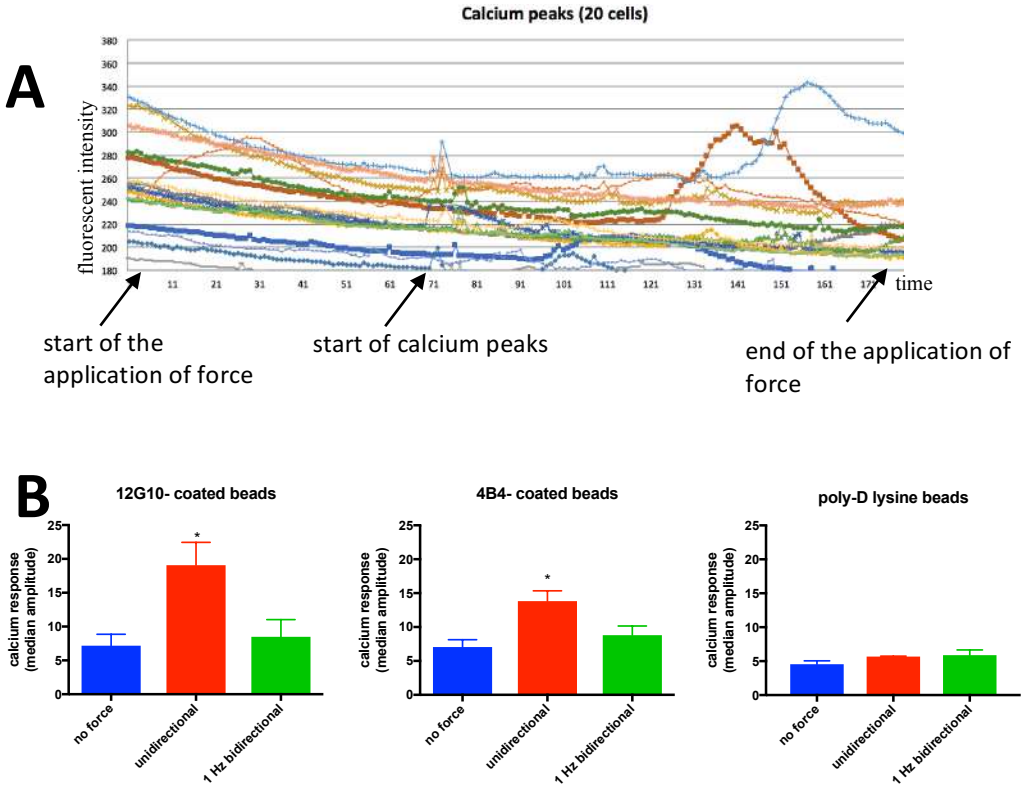


Figure 5.4 Application of unidirectional but not 1 Hz bidirectional force on either the active or inactive forms of β1- integrin induced calcium signalling

HUVECs were loaded with a calcium fluorescent dye (Cal- 520) and then incubated with beads coated non- covalently with 12G10 (targeting the active β1- integrin) or with 4B4 antibodies (targeting the inactive β1- integrin). As control, HUVECs were also incubated with beads coated covalently with Poly-D lysine. Unidirectional or 1 Hz bidirectional force (~16 pN) was applied onto the beads for 3 minutes and calcium responses were recorded using a fluorescent microscope. As a control, calcium responses in HUVECs incubated with beads but remained under no force were recorded for 3 minutes.

(A) Representative line graph shows individual calcium responses in relation to time of 20 cells incubated with 4B4- coated beads and experienced unidirectional force for 3 minutes. The first calcium responses occur around 75 seconds (indicated by the arrow) after the initiation of force.
(B) Quantification of calcium responses (median amplitude) showed a significant increase in calcium signalling in response to 12G10 or 4B4- coated beads exposed to unidirectional force. Unidirectional force on beads coated with Poly-D lysine failed to induce calcium responses. Furthermore, application of 1 Hz bidirectional force did not increase calcium signalling.
Mean values ± SEM are shown and statistical analysis was performed using a one- way ANOVA test, with Tukey's test for multiple comparisons (n=3-5, * indicates p=< 0.05).

5.2.3 The head region of β1- integrin responds to unidirectional force

It was shown that application of unidirectional force on either the inactive or the active β1- integrin induces calcium responses (Figure 5.4). I wanted to test whether application of unidirectional force on other regions of β1- integrin induces distinct calcium responses in endothelial cells.

Magnetic beads were therefore coated with antibodies targeting different regions of β1- integrin (Figure 5.3). HUVECs were loaded with a calcium fluorescent dye and then incubated with the beads. Unidirectional force (~16 pN) was applied on the beads for 3 minutes and the calcium responses were recorded using a fluorescent microscope. As a control, calcium responses of HUVECs incubated with beads but under no force were also recorded for 3 minutes.

Quantification of the calcium responses showed that only the beads targeting the head region of either the active or the inactive β1- integrin triggered a significant increase in calcium signalling under unidirectional force. On the contrary, application of unidirectional force on beads bound to other regions of the molecule, failed to induce a calcium response (Figure 5.5).

These data indicate that the head region only of β1- integrin can respond to unidirectional force and induce calcium responses.

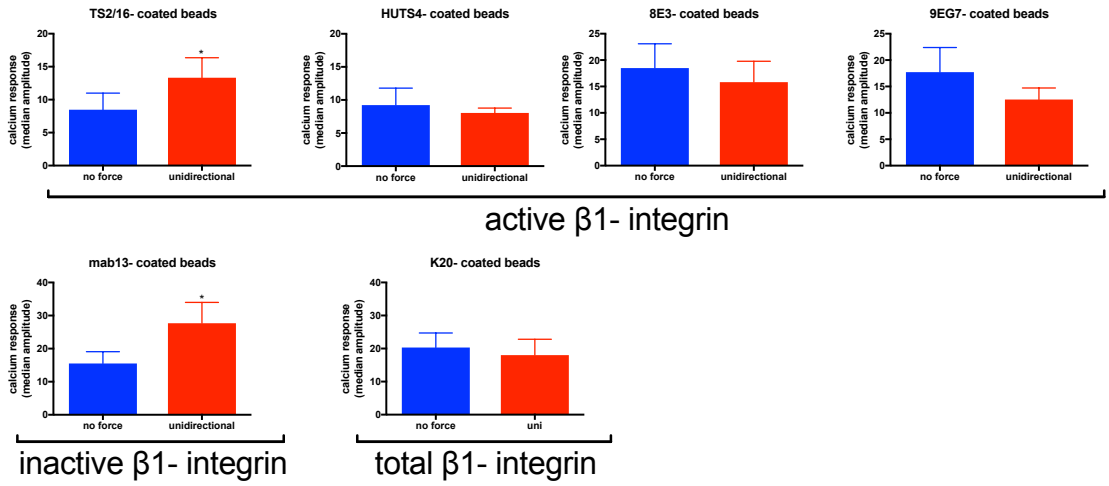


Figure 5.5 Application of unidirectional force on the head region only of β1- integrin induced calcium signalling

HUVECs were loaded with a calcium fluorescent dye and then incubated for 30 minutes with beads coated non- covalently with TS2/16, HUTS4, 8E3, 9EG7, mab13 and K20 antibodies, targeting different epitopes of the β1- integrin. Unidirectional force (~16 pN) was applied onto the beads for 3 minutes and calcium responses were recorded using a fluorescent microscope. As a control, calcium responses in HUVECs incubated with beads but remained under no force were recorded for 3 minutes.
Mean values ± SEM are shown. Differences were tested using a two- tailed, paired t test (n=5, * indicates p=< 0.05).
Quantification of calcium responses in responding cells showed that the head region only of either the inactive or the active β1- integrin responds to unidirectional force.

5.2.4 Phospholipase C regulates β1- integrin- mediated calcium responses under unidirectional force

I have previously shown that phospholipase C regulates β1- integrin-mediated calcium responses in endothelial cells under unidirectional flow (Figure 4.8). To examine whether phospholipase C regulates calcium responses in endothelial cells triggered by direct application of unidirectional force on β1- integrin, HUVECs were treated with phospholipase C inhibitor and unidirectional force was applied on beads bound to β1- integrin.

To be more specific, HUVECs were loaded with a calcium fluorescent dye and incubated with beads targeting the active β1- integrin (12G10- coated beads). Then, cells were treated with the phospholipase C inhibitor (U- 73122) or were left untreated and then exposed to unidirectional force for 3 minutes. Alternatively, HUVECs were treated with 10 μM of thapsigargin (depletes intracellular calcium stores) or were left untreated and then exposed to unidirectional force for 3 minutes to define the source of calcium. Calcium accumulation in the cytosol was recorded using a fluorescent microscope.

The quantification of calcium dynamics showed that treatment of cells with the phospholipase C inhibitor caused a significant decrease in calcium responses triggered by application of unidirectional force on β1-integrin compared to the untreated cells (Figure 5.6). Furthermore, treatment with thapsigargin totally abolished calcium increase caused by unidirectional force on β1- integrin.

These results suggest that the intracellular calcium stores and phospholipase C regulate β1- integrin- mediated calcium responses in response to unidirectional force.

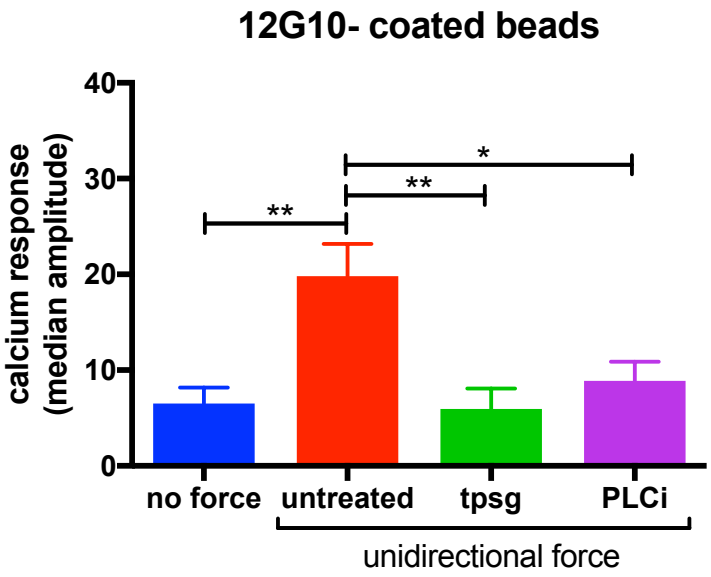


Figure 5.6 Depletion of intracellular calcium stores or inhibition of phospholipase C decreased β1- integrin- mediated calcium responses under unidirectional force

HUVECs were loaded with a calcium fluorescent dye (Cal- 520) and then incubated for 30 minutes with 12G10- coated beads, targeting the active β1- integrin. Then, cells were either treated with thapsigargin (tpsg) for 3 minutes or with phospholipase C inhibitor (U- 73122- PLCi) for 15 minutes. As a control, cells were left untreated. Unidirectional force (~16 pN) was applied for 3 minutes and calcium responses were recorded using a fluorescent microscope. As a control, calcium responses of HUVECs incubated with 12G10- coated beads but under force were also recorded.
Mean values ± SEM are shown. Differences were tested using a one- way ANOVA, with Tukey's test for multiple comparisons (n=5, * indicates p=< 0.05, ** indicates p=<0.01).
Quantification of calcium responses (median amplitude) showed a significant decrease in calcium signalling in cells treated with phospholipase C inhibitor or thapsigargin after the application of unidirectional force on 12G10- coated beads.

5.2.5 ROCK controls β1- integrin- mediated calcium responses under unidirectional force

Studies have shown that the cytoplasmic tails of β1- integrin interact with the actin cytoskeleton (Ross et al., 2013). As the cytoskeleton is also connected to the endoplasmic reticulum (Gurel et al., 2014), regulating some of its responses such as calcium release, we wanted to test whether the regulation of the cytoskeleton plays a role in transmission of signals triggered by mechanical stimulation of β1- integrin. Therefore, we examined the role of Rho-associated protein kinase (ROCK), a known regulator of the cytoskeleton, controlling the polymerisation of actin filaments (Amano et al., 2000).

First of all, I defined the baseline kinetics of calcium responses to β1-integrin mechanical activation. HUVECs loaded with a calcium fluorescent dye were incubated with beads coated with antibodies targeting either the inactive (4B4- coated beads) or the active β1- integrin (12G10- coated beads). Then, unidirectional force (~16 pN) was applied for either 30 or 60 seconds onto the beads and the calcium responses were recorded for 180 seconds using a fluorescent microscope. As a control, calcium signalling in HUVECs incubated with beads but under no force were also recorded. It was found that application of unidirectional force for 30 seconds failed to induce a significant increase in calcium signalling, whereas application of unidirectional force for 60 seconds (on either the inactive or the active β1-integrin) significantly enhanced calcium responses compared beads under no force (Figure 5.7 A). These data indicate that there is a minimum amount of time (60 seconds) required for the signal to be transmitted from the integrin at the apical surface to calcium stores.

In order to examine the potential role of ROCK, I inhibited it using the compound Y-27632. HUVECs were loaded with calcium fluorescent dye and incubated with 4B4- or 12G10- coated beads. Then, HUVECs were treated with the ROCK inhibitor or left untreated as a control.

Unidirectional force (~16 pN) was applied for 30 seconds and calcium dynamics were recorded using a fluorescent microscope. As a control calcium signalling of HUVECs incubated with beads but remained under no force were also recorded. The inhibition of ROCK in HUVECs led to a significant increase of calcium responses when unidirectional force was applied for 30 seconds on either the inactive or the active β1- integrin (Figure 5.7 B) compared to either untreated cells or cells under no force.

These data suggest that ROCK regulates β1- integrin- mediated calcium responses under unidirectional force.

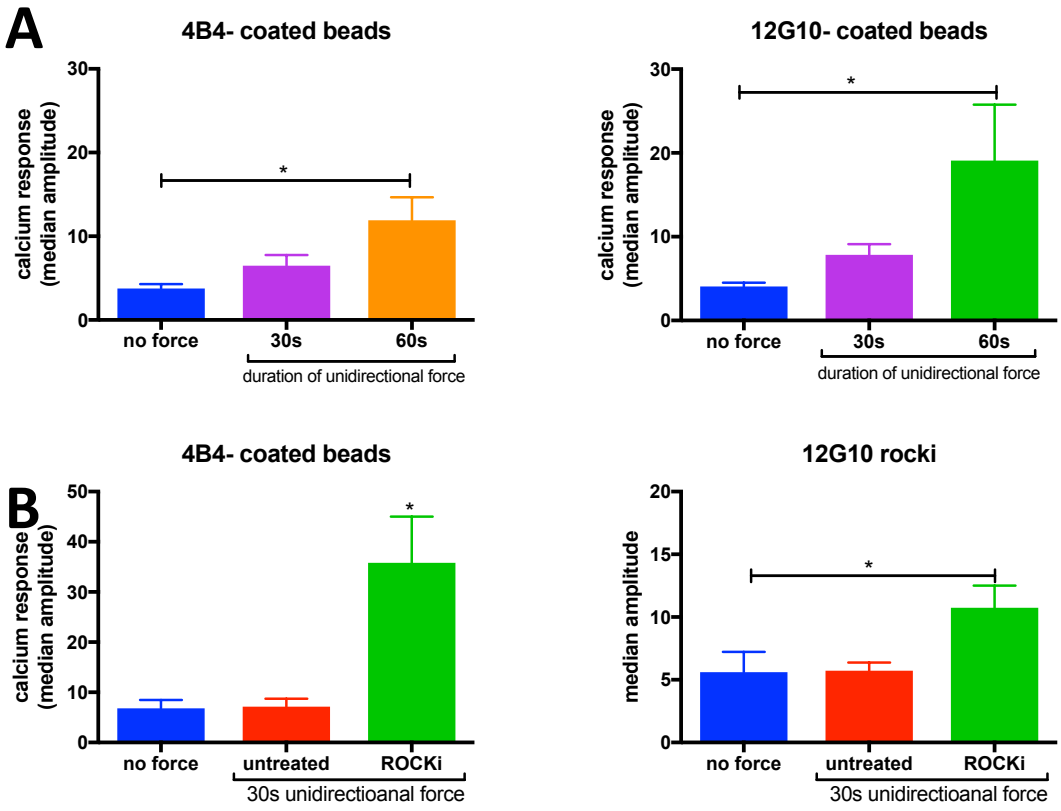


Figure 5.7 Inhibition of ROCK decreased the duration of unidirectional force required to induce β1- integrin- mediated calcium responses

(A) HUVECs were loaded with a calcium fluorescent dye and then incubated with beads coated non- covalently with 4B4 (top left) or with 12G10 antibodies (top right). Unidirectional force (~16 pN) was applied onto the beads for 30 or 60 seconds and calcium responses were recorded for 180 seconds using a fluorescent microscope. As a control, calcium responses in HUVECs incubated with beads but remained under no force were also recorded. Mean values ± SEM are shown. Differences were tested using a one- way ANOVA, with Tukey's test for multiple comparisons (n=3-5, * indicates p=< 0.05). Constant application of unidirectional force for 60 seconds on β1- integrin was required to induce a significant increase in calcium signaling in endothelial cells.
(B) HUVECs loaded with a calcium fluorescent dye (Cal- 520), incubated with beads coated non- covalently with 4B4 (bottom left) or with 12G10 antibodies (bottom right), were treated with 5 μM of ROCK inhibitor (ROCKi: Y-27632) for 10 minutes or left untreated. Unidirectional force (~16 pN) was applied onto the beads for 30 seconds and calcium responses were recorded for 180 seconds. As a control, calcium responses in HUVECs incubated with beads but remained under no force were also recorded. Mean values ± SEM are shown. Differences were tested using a one- way ANOVA, with Tukey's test for multiple comparisons (n=3-5, * indicates p=< 0.05).
Inhibition of ROCK significantly increased calcium responses triggered by application of unidirectional force for 30 seconds on β1- integrin.

5.3 Discussion

5.3.1 Conclusions

- β1- integrin responds to unidirectional but not 1 Hz bidirectional force by inducing calcium responses in endothelial cells
- The head region of β1- integrin is responsible for mechanosensation
- Phospholipase C regulates β1- integrin- mediated mobilisation of internal calcium stores
- There is a minimum time of unidirectional force on β1- integrin required to induce calcium signalling in endothelial cells
- ROCK regulates β1- integrin- mediated calcium responses in endothelial cells under unidirectional force

5.3.2 Discussion and future work

Mechanical activation of β1- integrin

This study has shown the direct mechanical activation of β1- integrin by specific mechanical forces (unidirectional force). β1- integrin is known to be activated by its interaction with components of the extracellular matrix and more specifically by the binding of its ligand (normally fibronectin) (Humphries et al., 2006). β1- integrin activation leads to disassociation of its cytoplasmic tail and regulation of downstream signalling pathways (Shyy and Chien, 2002). This project is the first to show the direct mechanical activation of the receptor in a ligand- independent manner, triggered by application of force and leading to structural changes. Other studies have shown that integrins can be activated indirectly by the application of force on other endothelial mechanoreceptors, such as PECAM- 1 (Collins et al., 2012). In addition, since the magnetic beads bind to the apical surface of the endothelial cells, our findings suggest that the receptor's activation takes place on the apical surface.

However, our findings do not rule out a role for the activation of β1- integrin on the basal surface of the cells, as mechanical forces are also exerted on that side and apical/basal responses are not mutually exclusive.

Our collaborator Dr. Antreas Kalli, a computational modeller from the University of Leeds, has studied the molecular mechanism linking force to integrin activation. β1- integrin is believed to have a very similar structure to β3- integrin (Ruoslahti, 1996, Xiong et al., 2001). As αvβ3- integrin's crystal structure is available, our collaborator performed some simulations and applied unidirectional force perpendicularly on the head region (labelled as orange on Figure 5.8) of the inactive β3 subunit of the avβ3 complex. He showed that β1- integrin unfolds in a stepwise manner until its full extension- activation (Figure 5.8). Further studies will be required to test these findings experimentally in a molecular level. One way to assess these data is the insertion of point mutations and the replacement of residues, crucial for the intramolecular rearrangements of the receptor. Furthermore, peptides that block the unfolding of the receptor could also be used to validate the model.

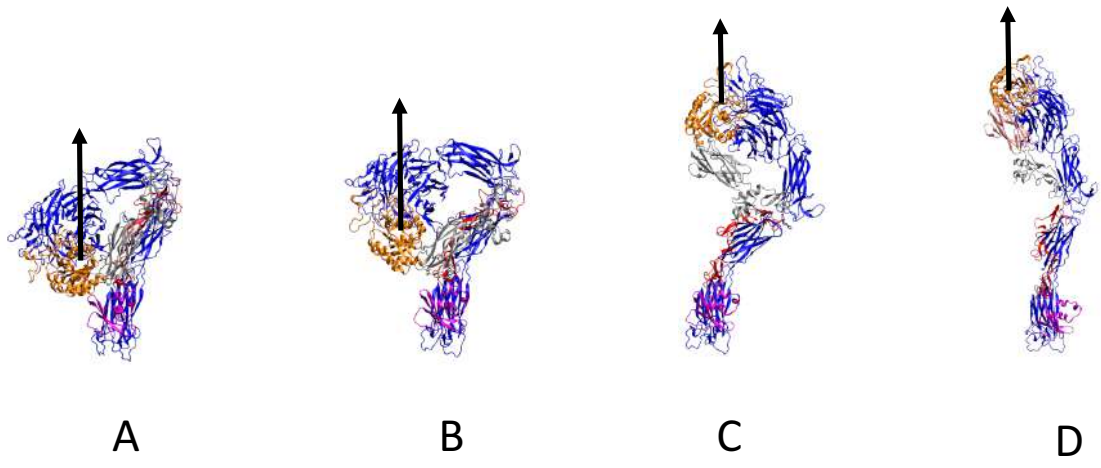


Figure 5.8 Computational simulation of β3- integrin activation by unidirectional force

Unidirectional force was applied perpendicularly (shown as black arrow) on the head region (labelled as orange) of the inactive β3- integrin (A). The constant application of force triggers intramolecular structural rearrangements (B and C) until the full extension of the molecule (D), which represents its active form.

Calcium responses in endothelial cells triggered by mechanostimulation of β1- integrin

Our data indicate that direct application of unidirectional but not 1 Hz bidirectional force on β1- integrin causes calcium responses in endothelial cells. Other studies have shown that the mechanical stimulation of other mechanoreceptors, such as primary cilia and Piezo1, enhances calcium responses in endothelial cells (Nauli et al., 2008, Li et al., 2014). However, these studies did not compare the application of mechanical forces with different directionality. Our findings confirm that β1- integrin responds to unidirectional force only. Future work can include the application of other force patterns, such as bidirectional force, on other mechanoreceptors (piezo1, trpv4 etc).

Using antibodies targeting different structural regions of β1- integrin, it was revealed that the application of unidirectional force on the head region only triggers calcium responses in endothelial cells. One possible explanation of this finding is that the molecule needs to have a minimum length in order to be able to sense the mechanical stimulus and transduce the signal, triggering downstream pathways. Thus, the application of force on its head, at the membrane- distal end of the molecule, is sufficient to promote calcium responses, whereas the mechanostimulation of intermediate regions, fails to induce calcium signalling. Other studies support this hypothesis as they have shown that the length of other mechanoreceptors, such as primary cilia, affect their function and ability to sense mechanical forces (McGlashan et al., 2010, Goetz et al., 2014). Therefore, application of mechanical force on the head of β1- integrin may lead to deflection of the extended molecule and as a result β1- integrin acts as a molecular “lever”. Further studies can include the deletion of specific regions on β1- integrin that will shorten the molecule and then test the cellular responses after the mechanical stimulation of the receptor.

The role of the cytoskeleton in transduction of mechanical signals on β1- integrin

This project showed that the depletion of the inner calcium stores with thapsigargin led to decreased calcium responses when force was applied on β1- integrin. In addition, inhibition of Phospholipase C, a molecule regulating calcium release from the inner stores, also reduced the calcium signalling triggered by direct mechanostimulation of β1- integrin. These two findings suggest that the observed calcium signals triggered by application of unidirectional force on β1- integrin originate from endoplasmic reticulum stores. On the contrary, a study by Matthews suggests that the application of force on β1- integrin triggers the activation and subsequent opening of the TRPV4 channel, leading to calcium influx into the cell (Matthews et al., 2010). As a result, that study proposes that β1- integrin and TRPV4 form a complex, with β1- integrin to be the mechanosensor and TRPV4 the channel responsible for calcium influx. The most significant difference between my observation and their data is the amount of applied force. In my study, mechanical forces of ~16 pN were applied on magnetic beads, mimicking the magnitude of shear stress. On the contrary, the experiments by Matthews include the use of a magnetic needle generating forces around 2000 pN, a magnitude of force similar to those exerted from the extracellular matrix on integrins based on the basal surface of the cells (Schwartz, 2010). Therefore, the activation and opening of TRPV4 by mechanical stimulation of β1- integrin may be caused by mechanical forces similar to those exerted on the basal surface of the endothelial cells, such as mechanical stretch, whereas shear forces may be insufficient to initiate β1- integrin- TRPV4 crosstalk.

In addition, the calcium responses observed by Matthews in that study were localised around the beads, whereas I studied the calcium signalling in whole cells after the application of force on the mechanoreceptor. Finally, the magnetic beads of the Matthews' study were coated with fibronectin, a ligand binding to both the a and β subunit of several integrin complexes and not only to β1- integrin, whereas the beads used in the current study were coated with antibodies targeting β1- integrin specifically (Humphries et al., 2006). It is therefore plausible that mechanical activation of the α subunit is required for TRPV4 channel opening.

My data indicate that there is a minimum time (60 seconds) of applied force required for the mechanical signal to be transduced, starting from the stimulation of β1- integrin on the apical surface and through the cytoskeleton to reach the inner calcium stores, causing calcium release. The fact that there is no significant difference in this time when force is applied on either the inactive or the active β1- integrin suggests that the unfolding of the receptor is rapid. In addition, ROCK seems to play a crucial role in the transduction of the signal through the cytoskeleton, since its inhibition accelerated the transmission of the signal. It is known that the actin cytoskeleton is a dynamic network of actin filaments, microtubules and intermediate filaments (Wang et al., 1993, Ingber, 1993). The endothelial cytoskeleton maintains its strain and reorganises itself when the cell migrates, proliferates or is under the effect of fluid mechanical forces (Helmke and Davies, 2002, Hsiao et al., 2016). ROCK regulates the organisation of the actin cytoskeleton, by inhibiting the depolymerisation of the actin filaments and thus maintains the cytoskeletal strain (Noma et al., 2006, Marjoram et al., 2014). Therefore, its pharmacological inhibition will cause the depolymerisation of the actin cytoskeleton and subsequently the loss of the cytoskeletal strain.

My findings demonstrate that the pre- treatment of endothelial cells with ROCK inhibitor decreased the duration of the application of unidirectional force, which is required in order to induce calcium responses. Data from other studies support this observation, as the treatment of endothelial cells with cytochalasin D, a non- specific inhibitor of actin polymerization, caused a significant increase in calcium signalling triggered by mechanical stimulation of integrins (Matthews et al., 2010). However, studies have shown that the use of Y27632 (the ROCK inhibitor also used in the current project) leads to partial inhibition of Ca^{2+} sensitization in some cell types, such as smooth muscle cells, indicating that other pathways independent of ROCK are associated in calcium signalling. In addition, the efficiency of the inhibitor may be associated with the mechanical properties of the cells (Fu et al., 1998). Therefore, the use of siRNAs, which will knock down ROCK specifically, may serve as an alternative approach in order to achieve specificity. My project highlights the importance of a specific molecule, ROCK, in the transmission of mechanical signals from the apical surface to the cytoskeleton. Future studies could include the investigation of downstream targets of ROCK, such as LIM kinases, and their role in endothelial mechanotransduction.

Chapter 6: General Discussion

6.1 Main conclusions

- Calcium responses in endothelial cells were flow specific. The source and magnitude of calcium signalling was dependent on the direction of flow. Application of unidirectional flow caused calcium release from the intracellular stores, whereas 1 Hz bidirectional flow triggered both extracellular calcium influx and calcium release with intracellular origin.
- β1- integrin was activated in endothelial cells exposed to unidirectional but not bidirectional flow. The activation of β1- integrin was found to be triggered by direct application of force on the molecule.
- β1- integrin induced calcium responses specifically to unidirectional but not to 1 Hz bidirectional flow. Calcium responses were triggered when unidirectional force was applied directly on the head region of β1- integrin causing the transition from the folded to the unfolded form of the molecule. Phospholipase C and ROCK were found to mediate the calcium responses induced by β1- integrin mechanostimulation.

6.2 β1- integrin is a mechanoreceptor required for endothelial responses under unidirectional flow

The role of several mechanoreceptors regulating endothelial responses to flow has already been described in the literature. The current project aimed to investigate whether a single mechanoreceptor can sense different blood flow patterns or different receptors are required for each specific condition. The data obtained in this study has identified β1- integrin as a mechanoreceptor based on the apical surface of endothelial cells, which responds to unidirectional force only.

Therefore, a model of endothelial responses regulated by distinct mechanoreceptors under the effect of different flow patterns can be proposed (Figure 6.1). Unidirectional shear stress activates β1- integrin on the apical surface of endothelial cells. Although not tested here, it is also plausible that basal β1- integrin can be activated specifically by unidirectional force. This mechanical activation leads to calcium release from the intracellular calcium stores and is regulated by phospholipace C and ROCK. The mechanical stimulation of β1- integrin also regulates chronic endothelial responses to unidirectional flow, such as cytoskeletal alignment to the directional of flow. These responses have been shown to promote atheroprotection (Baeyens et al., 2014). On the contrary, endothelial responses under bidirectional shear stress are regulated by yet unknown mechanoreceptors. Several mechanoreceptors, such as the glycocalyx, have been proposed to regulate responses to bidirectional/ disturbed flow (Cancel et al., 2016). However, there are no convincing evidence to support the hypothesis that a single mechanoreceptor can sense bidirectional shear stress directly. The data of the current study showed that calcium signalling under bidirectional flow has both extracellular and intracellular origin. Therefore, it would be reasonable to hypothesize that a potential mechanoreceptor regulating responses under bidirectional flow could be a mechano- gated calcium channel on the plasma membrane, which allows the influx of calcium from the extracellular space. Some candidate calcium channels could be Piezo-1 or TRPV4, which have been previously show to respond to mechanical forces (Mendoza et al., 2010, Li et al., 2014). The influx of calcium through these channels may induce an atherosusceptible endothelial phenotype.

As a result, the current project elucidates the mechanisms that decipher the mechanical code in endothelial cells and gives the opportunity to better understand vascular pathophysiology.

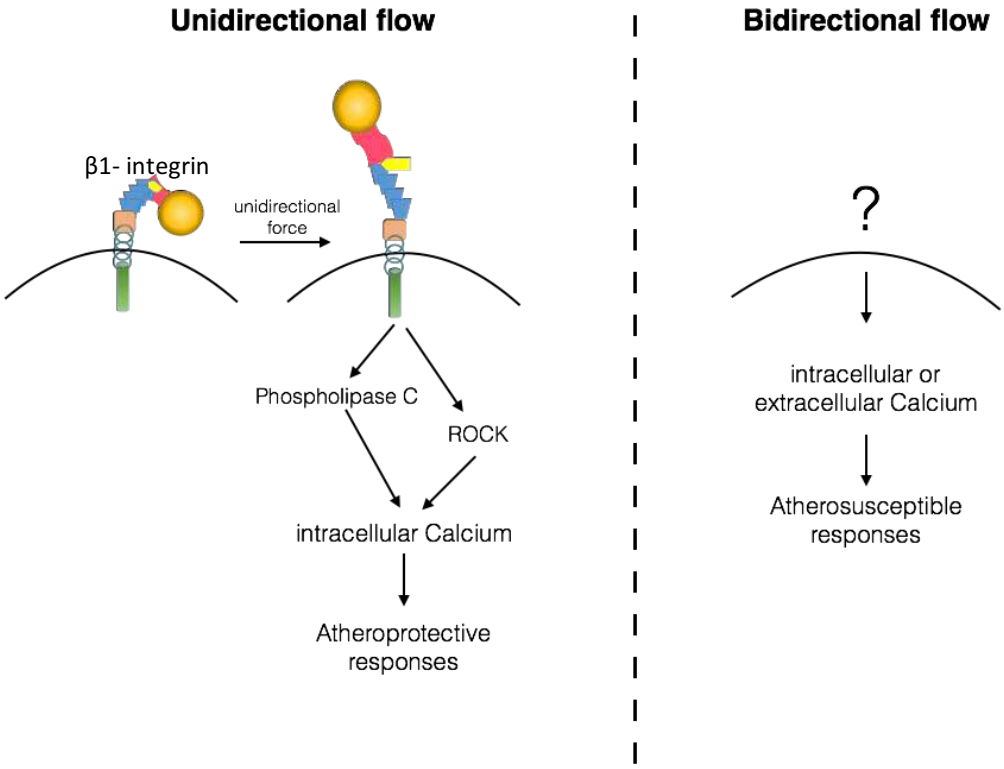


Figure 6.1 A proposed model of β1- integrin mediated mechanoresponses in endothelial cells under different flow patterns

Unidirectional flow triggers mechanical activation of β1- integrin. This mechanical activation leads to calcium release from the intracellular calcium stores. Calcium signalling is regulated by Phospholipase C and ROCK. The mechanical activation of β1- integrin promotes atheroprotective responses, such as cytoskeletal alignment to the direction of flow.
Although calcium responses are induced under bidirectional flow, β1- integrin is not activated by this flow pattern. Therefore, another unknown mechanoreceptor, which allows both calcium influx from the extracellular space and release from the intracellular stores, facilitates endothelial responses leading to atherosusceptible phenotypes.

6.3 Limitations of this study

Although the current thesis elucidates the mechanism by which endothelial cells sense different flow patterns, it must be acknowledged that there are certain limitations in this study.

First of all, the majority of experiments were performed *in vitro* using HUVECs. On the one hand, the use of HUVECs can be high throughput because they are readily available, allowing the repetition of several experiments and taking into account the genetic variability of different donors from who cells were isolated. On the other hand, their venous origin does not replicate the endothelial cell types involved in the development of atherosclerosis. Therefore, the use of arterial cells, such as human coronary arteries endothelial cells, would be more suitable as atherosclerosis develops within the arterial system. However, a study by Aranguren suggests that the identity of both arterial and venous isolated endothelial cells is less distinct after their *in vitro* culture for several weeks (Aranguren et al., 2013). Thus, the use of HUVECs can provide a useful starting point to study molecular processes involved in atherosclerosis.

Although either the flow system or the magnetic tweezers platform used in this study are *in vitro* systems, which can simulate shear stress patterns of atheroprotective or atheroprone conditions, they do not completely replicate flow patterns occurring *in vivo.* In addition, these *in vitro* systems can incorporate only cultured endothelial cells and not other cell types, such as vascular smooth muscle cells (VSMCs), which can be crucial for the initiation and progression of atherosclerosis. Endothelial cells exposed to shear stress cross- talk with underlying VSMCs, regulating their responses (Qiu et al., 2014). This interaction can change the phenotype of both endothelial and VSMCs (Qi et al., 2011). Moreover, the composition of extracellular matrix (ECM) *in vivo* differs to what was as a substrate of endothelial cells *in vitro*. Extracellular matrix is consisted of collagen, elastin, laminin, fibronectin etc (Bosman and Stamenkovic, 2003).

The current project included the use of fibronectin as a substrate of HUVEC. However, some of the described experiments on calcium dynamics were repeated with the use of gelatin (data not shown) and there were no significant differences compared to those where fibronectin was used. The composition of the ECM can be crucial for the mechanotransduction and affect the mechanical properties of the cells (Jalali et al., 2001, Orr et al., 2005, Chen et al., 2017). Thus, the endothelial cell behaviour can be different between an endothelial monolayer cultured *in vitro* compared to arteries *in vivo*.

Finally, the experiments performed in this thesis did not take into account other mechanical forces, which may be applied in cells *in vivo*. To be more, specific, endothelial cells are under the effect of shear stress, pressure and stretch (Davies and Tripathi, 1993). The cells have developed mechanisms to respond to all of these mechanical stimuli and they may coordinate in order to determine endothelial cell behaviour. As a result, shear stress, which has been studied thoroughly in this thesis, is not the only force affecting cells and *in vivo* experiments could provide a more extensive study of distinct mechanical forces affecting endothelial cells.

In summary, the majority of these limitations can be adressed by using an *in vivo* model, such as an endothelial- specific β1- integrin knock out mouse or a zebrafish morpholino against β1- integrin, as this would elucidate the role of β1- integrin in vascular pathophysiology.

6.4 β1- integrin as a therapeutic target

This study has shown that the activation status of β1- integrin affects endothelial responses to different flow patterns. Thus, its pharmacological-induced activation may promote atheroprotection. Indeed, a study by Wang has shown that the oral administration of $MnCl_2$, a known activator of β1-integrin, reduced the formation of plaques in *ApoE*$^{-/-}$ mice on Western diet for 12 weeks (Wang et al., 2013). However, $MnCl_2$ is not a specific activator of β1- integrin and this finding may be a result of off- target effects. Therefore, our data in conjunction with results of the computational modelling of β1- integrin activation, may indicate residues crucial for the activation of the molecule. Future therapeutic applications could include drugs targeting these specific residues, triggering the activation and maintaining the molecule on this status.

Moreover, integrins play an important role in other processes of the cardiovascular system, such as angiogenesis (Hynes, 2002). In some pathological cases, such as myocardial ischemia, the enhancement of myocardial angiogenesis is required in order to regenerate the injured tissue. A study by Hatanaka, using low- energy shock wave therapy - a method to improve myocardial ischemia - has found that the mechanical activation of β1- integrin led to phosphorylation of Erk and Akt, two molecules involved in angiogenic signalling pathways (Shiojima and Walsh, 2002, Mavria et al., 2006, Hatanaka et al., 2016). Therefore, the mechanical activation of β1- integrin, using shock waves for example, seems to play a beneficiary role in some cases of cardiovascular diseases.

Finally, the quick re- endothelialisation of stented arteries is an essential step after arterial injury, caused by the placement of the stent (Versari et al., 2007). However, the migration of the endothelial cells into the stent may differ and depend on the surrounding flow patterns (Hsiao et al., 2016).

As β1- integrin has been shown to affect endothelial cell migration, its pre-activation, localised around the area of the stent with the use of a stent eluting $MnCl_2$ or coated with antibodies activating β1- integrin, may accelerate cell migration and provide quicker recovery of the stented artery (Senger et al., 2002).

In summary, β1- integrin and especially its activation may be a novel therapeutic target for the treatment of several cardiovascular diseases. However, the activation of β1- integrin as a goal of therapy should be approached with caution, as its excessive activation may lead to carcinogenesis (Desgrosellier and Cheresh, 2010).

6.5 Future studies

Although this study is the first to show that β1- integrin, a single mechanoreceptor, can distinguish different applied flow patterns and consequently change its activation status, further investigation is required as many questions remain unanswered.

Application of unidirectional flow was found to activate β1- integrin, which then led to calcium release from the intracellular stores. This finding is important as it gives an insight of how the function of a mechanoreceptor based on the apical surface of the cells is linked with downstream signalling pathways, activated by specific forces. However, the intermediate molecular events between β1- integrin mechanical activation and calcium release require further investigation. To be more specific, it is known that activation of β1- integrin triggers the disassociation of its cytoplasmic tails and subsequently the recruitment of talin and engagement of vinculin (Das et al., 2014, Yao et al., 2014). This whole process is mechanically driven and leads to reorganisation of the cytoskeleton (Morse et al., 2014). Although my data indicate that the activation status of β1- integrin affects cytoskeletal organisation under flow, the exact mechanism remains unclear.

Future work can include the silencing of molecules crucial for this mechanotransduction pathway, such as talin and vinculin and then the application of force on β1- integrin. Furthermore, the development of fluorescence resonance energy transfer (FRET) sensors for specific molecules involved in this pathway will enable the measuring of mechanical tension and identify possible structural rearrangements. FRET sensors have been extensively used in the field of vascular mechanobiology and have provided useful information regarding the transmission of mechanical forces into the cells (Grashoff et al., 2010, Liu et al., 2013).

Recent evidence suggests that several endothelial mechanoreceptors cross- talk in order to regulate cell behaviour under flow (Matthews et al., 2007, Collins et al., 2012). The current study focused solely on the contribution of β1- integrin to endothelial mechanoresponses. However, as different mechanoreceptors coordinate and may form a network, it would be useful to follow a broader approach and test the interaction of different mechanoreceptors (Givens and Tzima, 2016). For instance, studies have shown that mechanical activation of PECAM- 1 leads to integrin activation (Tzima et al., 2005(Collins et al., 2012, Collins et al., 2014). Another study suggested that application of force on integrins led to activation of TRPV4 channels (Matthews et al., 2010). Therefore, it would be interesting to silence some of these mechanoreceptors, such as PECAM-1, and then apply force directly on β1- integrin using the magnetic tweezers platform and test the effects on endothelial cell behaviour. As endothelial cells form a confluent monolayer and force is applied on the apical surface, the absence of a crucial mechanoreceptor (PECAM-1) located on the intercellular junctions, may have a significant effect on the transmission of the mechanical signals. As a result, this work can be extended to other mechanoreceptors, some of which can be found to regulate responses to bidirectional flow.

The idea of testing the role of individual mechanoreceptors *in vivo* has already been used from other researchers in the field of vascular biology. A study by Chen suggested that the absence of other mechanoreceptors, such as PECAM- 1, in knockout mice causes impaired vascular remodelling and blocks flow- induced signalling pathways, such as NF- κB activation under oscillatory shear stress (Chen and Tzima, 2009). Therefore, the investigation of the role of β1- integrin *in vivo* would be very useful. However, as the β1- integrin knockout mouse is developmentally lethal, the use of a conditional endothelial- specific β1- integrin knockout mouse would help to highlight the importance of this mechanoreceptor. This study provided *in vitro* evidence that β1- integrin is required to sense unidirectional flow and its inhibition leads to endothelial dysfunction. Therefore, it would be reasonable to hypothesize that endothelial cells of knockout mice lacking β1- integrin will have lost their ability to sense and ultimately respond to unidirectional flow. One possible experiment could be the assessment of endothelial cell alignment or the expression of inflammatory markers in the descending aorta of knockout mice, which is considered to experience high shear stress laminar flow. If β1- integrin is essential for sensing unidirectional flow *in vivo* too, animals lacking endothelial β1- integrin will have impaired alignment of endothelial cells, as well as increased expression of inflammatory markers in the descending aorta. Furthermore, if β1- integrin plays a role in the initiation and progression of atherosclerosis, then the endothelial- specific β1- integrin knockout mice fed with high fat diet would develop atherosclerotic plaques in regions, which are normally thought to be atheroprotective such as the descending aorta or the outer curvature of the aortic arch. An animal model, allowing high throughput studies, could also be zebrafish, which has been previously used to study not only angiogenesis but also atherosclerosis (Watson et al., 2013, Mahmoud et al., 2016, Menon et al., 2016).

However, zebrafish model has 4 orthologues of β1- integrin and their simultaneous silencing in order to avoid compensation would be challenging (Mould et al., 2006).

In summary, this study expanded the existing knowledge of how endothelial cells sense different flow patterns. Since endothelial cells behaviour is determined by their ability to sense the surrounding flow patterns, further research into the role of β1- integrin could lead to the development of a novel therapeutic application against several cardiovascular diseases, including atherosclerosis.

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