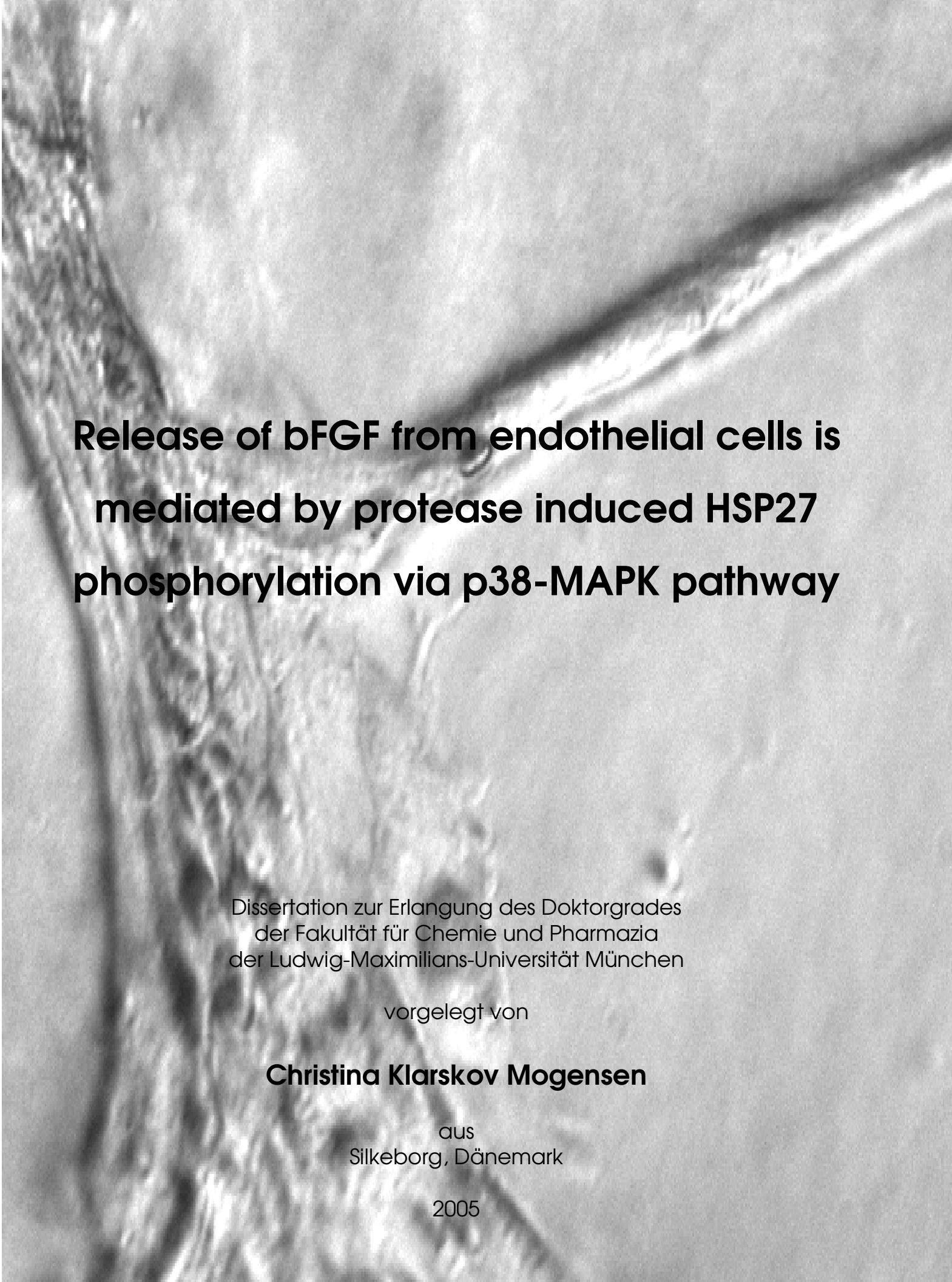


Release of bFGF from endothelial cells is mediated by protease induced HSP27 phosphorylation via p38-MAPK pathway

Dissertation zur Erlangung des Doktorgrades
der Fakultät für Chemie und Pharmazia
der Ludwig-Maximilians-Universität München

vorgelegt von

Christina Klarskov Mogensen

aus
Silkeborg, Dänemark

2005

Erklärung

Diese Dissertation wurde im Sinne von §13 Abs.3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von Herr Prof. Dr. Ulrich Pohl, und von Frau Prof. Dr. Angelika M. Vollmar von der Fakultät für Chemie und Pharmazie betreut.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbstständig, ohne unerlaubte Hilfe erarbeitet.

München, 19. Dezember 2005

Christina Klarskov Mogensen

Dissertation eingereicht am 19.12.05

1. Gutachter: Herr Prof. Dr. Ulrich. Pohl
2. Gutachter: Frau Prof. Dr. Angelika. M. Vollmar

Mündliche Prüfung am 30.01.06

To my parents, my son, Victor, and my husband, Henrik

Abstract

Introduction: Factors and other stimuli that lead to the release of basic fibroblast growth factor (bFGF) from endothelial cells may be essential for physiological processes such as development and angiogenesis. The release mechanisms are somewhat obscure and it has previously been shown that in the case of shear stress induced bFGF release cell matrix interaction is critically mediating that bFGF release (Gloe *et al.*, 2002). Considering the potential role of proteolytically modified extra-cellular matrix components in the induction of cellular signaling cascades, the aim of the present study was to investigate whether elastase activity contributes to the release of bFGF from endothelial cells.

Methods and results: Treatment of porcine aortic endothelial cells with elastase led to a release of bFGF in a concentration-dependent manner. This release was strictly regulated and could be reduced by inhibition of integrin $\alpha_v\beta_3$. Moreover, bFGF was translocated towards the cell membrane after elastase treatment as well as shear stress exposure, in close proximity to HSP27. Furthermore, elastase treatment led to a p38 MAP Kinase dependent HSP27 phosphorylation and this phospho-HSP27 could be shown to co-precipitate with bFGF.

Conclusion: We conclude that elastolytic activities activated by shear stress are involved in the active release of bFGF from endothelial cells and that phosphorylation of HSP27 is prerequisite for this release mechanism. The results may reflect the critical role of proteases in the initial process of angiogenesis induction.

Content

Abbreviation

A:	Ampere
ABC:	ATP-binding cassette
Abx:	Abciximab
App:	Approximately
AP-1:	Activator protein-1
APS:	Ammoniumpersulfate
BAEC:	Bovine Aorta Endothelial Cells
BCA:	Bicinchoninic Acid
bFGF:	Basic fibroblast growth factor
BSA:	Bovine serum albumin
Da:	Dalton
DAG:	Diacylglycerol
dH_2O:	Distillated water
DMEM:	Dulbecco's modification of Eagle's medium
ECL:	Enhanced Chemoluminescence reagent
ECM:	Extracellular matrix
Ela:	Elastase
ELISA:	Enzym-linked immunosorbe
ER:	Endoplasmatic reticulum
ERK:	Extracellular signaling-regulated kinase
FACE:	Fast activated cell based ELISA
FACS:	Fluorescence activated cell sorter
FAK:	Focal adhesion kinase
FCS:	Fetal calf serum
FGFR:	bFGF receptor
FITC:	Fluoresceinisothiocyanate
FRS2:	FGF receptor substrate 2
GTP:	Guanosine triphosphate
H_2O_2:	Hydrogen peroxide
HRP:	Horseradish peroxidase

HS:	Heparan sulfate
HSP:	Heparan sulfate proteoglycan
HSP27:	Heat shock protein 27
HUVEC:	Human Umbilical Vein Endothelial cells
IEF:	Isoelectric focusing
IG:	Immunoglobulin
JNK:	c-Jun NH_2-terminal protein kinase
L:	Liter
L-15:	Leibovitz medium
m:	Milli
M:	Molar
MAPK:	Mitogen activated protein kinase
MAPKAPK:	Mitogen activated protein kinase activated protein kinase
Min:	Minute
mRNA:	messenger Ribonucleinic acid
n:	Nano
NF-κB:	Nuclear Factor κB
NOS:	Nitric oxide synthase
NS:	Non-significant
OD:	Optical Density
p:	Pico
PACE:	Phosphospecific antibody cell-based ELISA
PAEC:	Porcine Aorta Endothelial Cells
PAGE:	Polyacrylamide-gel electrophoresis
Perm:	Permeable
PBS:	Phosphate-buffered saline
PECAM:	Platelet/endothelial cell adhesion molecule
PI:	Propidium iodide
PIP2:	Phosphatidylionsitol 4,5-biphosphate
PKC:	Protein kinase C
PLCγ:	Phospholipase Cγ
PTK:	protein tyrosin kinase
rpm:	Rounds per minute

PRAK:	p38 regulated and –activated protein kinase
RT:	Room temperature
µ:	Micro
SDS:	Sodium dodecyl sulfate
Sec:	Second
SEM:	Standard error of the mean value
SRP:	Signal-recognition particle
TEMED:	N,N,N',N'-tetramethylethylendiamine
TK:	Tyrosine kinase
TBS-T:	Phosphate buffered saline solution with Tween 20
TGF-β3:	Transforming growth factor-beta 3
TM:	Transmembrane
TNF-α:	Tumor necrosis factor alpha
Tris:	Tris-hydrozymethyl-aminomethan

Introduction

Background

Angiogenesis is a fundamental process in reproduction and wound healing and is prerequisite in placental development and tumor growth (Liekens *et al.*, 2001;Prior *et al.*, 2004). It is a complex process consisting of different sequential steps, which include the release of proteases from endothelial cells with subsequent degradation of the basement membrane surrounding the pre-existing vessels, migration of endothelial cells into the interstitial space, endothelial cell proliferation and differentiation into mature blood vessels. For these processes to proceed, an extensive interplay between cells, angiogenic inducers and extracellular matrix components is necessary. Among angiogenic factors are the "basic fibroblast growth factor" (bFGF) and "vascular endothelial growth factor "(VEGF) the most important and best characterized (Liekens *et al.*, 2001).

VEGF is expressed in different tissues and by many cells and it acts specifically on endothelial cells (Veikkola & Alitalo, 1999). *In vitro*, VEGF stimulates degradation of the extracellular matrix, and migration, proliferation, and tube formation of endothelial cells. *In vivo*, the VEGF has been shown to regulate vascular permeability, which is essential for the initiation of angiogenesis (Liekens *et al.*, 2001).

Like VEGF, bFGF induces processes *in vitro*, which are critical for angiogenesis. Basic FGF stimulates endothelial cell proliferation, migration and production of urokinase-type plasminogen activator, and it induces tube formation in collagen gels (Papetti & Herman, 2002; Liekens *et al.*, 2001). In contrast to VEGF, bFGF does not only act on endothelial cells, but stimulates proliferation of almost all cell types (Papetti & Herman, 2002). Furthermore, bFGF is not only important in angiogenesis, but is a pleiotropic protein that can exert multiple functions on a variety of cells (Bikfalvi *et al.*, 1997; Nugent & Iozzo, 2000; Chen *et al.*, 2004). Basic FGF is distinguished from most other proteins by the fact that it lacks the signal sequence needed for classical protein export from cells. Hence, the release of the protein still needs to be elucidated, which makes bFGF a unique and very interesting protein.

Basic fibroblast growth factor (bFGF)

In the early 1970s, Gospodarowicz and his co-workers purified a protein from bovine pituitary gland, which was capable of inducing proliferation and phenotypic transformation of BALB/c 3T3 fibroblast (Gospodarowicz, 1974; Gospodarowicz *et al.*, 1974; Gospodarowicz, 1975). This protein was named "basic fibroblast growth factor" (bFGF), because of its basic isoelectric point of 9.6. Some years later another FGF-related protein was recognized and named acidic FGF (aFGF), again, based upon its isoelectric point of 5.6 (Miller *et al.*, 2000). Since then, many other FGFs have been purified and cloned and in 1991, when identification of 7 FGFs had been made, the aFGF and bFGF were renamed to FGF-1 and FGF-2, respecitively, however the original names of the two first isolated FGFs were preserved for historical reasons (Chen *et al.*, 2004). At the same time it was emphasized that the name FGF did not mean that the major task of these protein was stimulation of fibroblast proliferation. To date 22 members of the FGF family have been identified in humans and vertebrates (Ornitz & Itoh, 2001).

In the following I will address structural, chemical and functional properties of bFGF and focus on its non-classical secretion from the producing cells.

Structure

Since 1975, the primary structure of bFGF has been sequenced and its cDNA cloned (Abraham *et al.*, 1986b). The sequence homology for bFGF across a wide range of species is very high (>90%) (Nugent & Iozzo, 2000), in particular, the sequence of human bFGF is 99% homologous to that of the bovine bFGF (Abraham *et al.*, 1986b). The human genome has only one copy of the bFGF gene (*fgf2*), which has been mapped to band q26-q27 on the human chromosome 4 (Dow & deVere White, 2000; Okada-Ban *et al.*, 2000). The size of the gene is 40 kilobases and the coding sequence consists of three exons, which are interrupted by two introns (Abraham *et al.*, 1986b) (figure 1A). *Fgf2* is a unique gene and differ from most eukaryotic genes in the way that it lacks the CAAT and TATA box motifs found in the promoters of most genes (Shibata *et al.*, 1991). Instead its promoter contains activator protein-1 (AP-1)

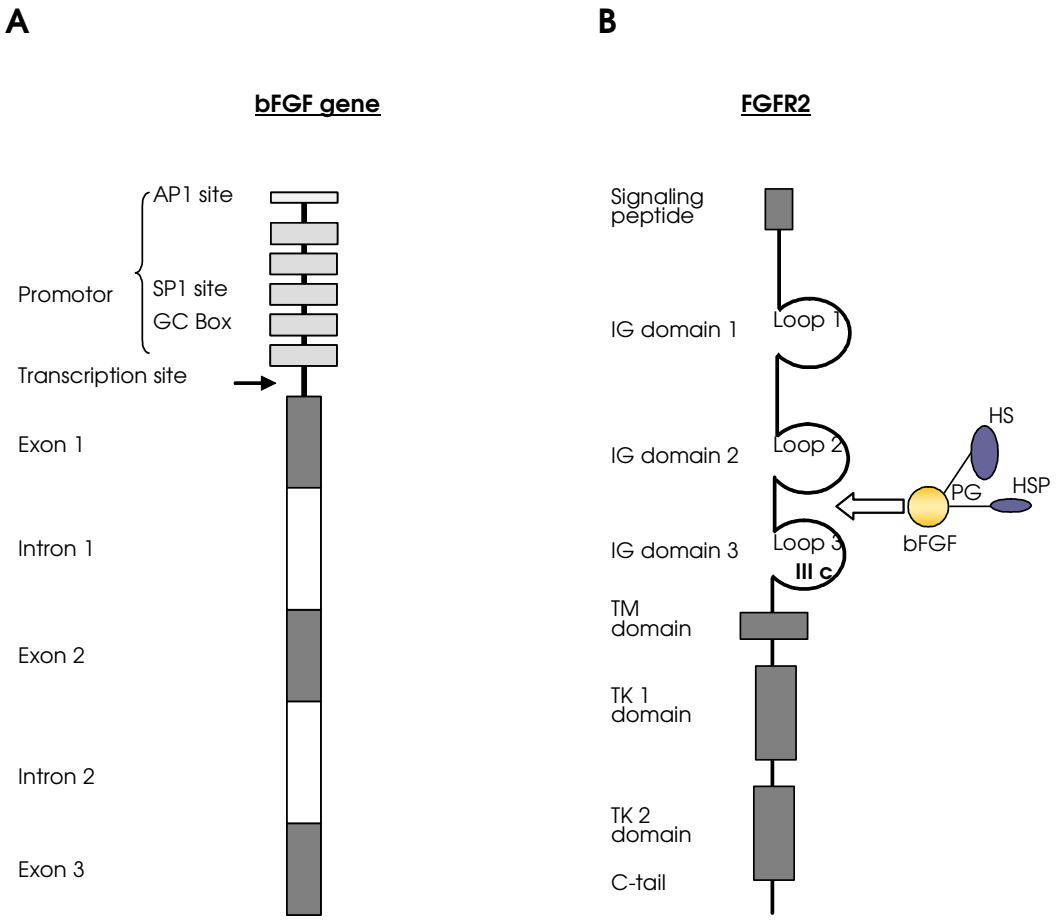


Figure 1: Schematic representation of the structure of bFGF (A) and its receptor, FGFR2 (B). (A) The bFGF gene does not have CAAT or TATA boxes, but consists instead of the promoter, which includes a transcription factor AP-1 (activator protein-1) and five GC boxes forming a SP-1 binding site. (B) The bFGF receptor has three extracellular immunoglobulin (IG) domains, a transmembrane (TM) domain and two intracellular tyrosine kinase (TK1-2) domains. Note that bFGF binds to the IIIc isoform of the IG loop3. The binding affinity of bFGF for its receptor increases when proteoglycans (PG), eg. heparan sulfate (HS) and heparan sulfate proteoglycans (HSP), are bound to bFGF ligand. Modified after Dow & deVere White, 2000.

binding sites and five GC boxes, but the functional relevance of these sites is not yet defined (Chen *et al.*, 2004).

An AUG codon on the bFGF cDNAs was found to initiate translation of a protein of 155 amino acids and no in frame AUG codons were found upstream (Bikfalvi *et al.*, 1997). Hence, the translation was predicted to initiate at this AUG codon. However, higher molecular weight forms (196, 201 and 210 amino acids) were found later (Bikfalvi *et al.*, 1997), and recently an even longer isoform has been identified (288 amino acids) (Arnaud *et al.*, 1999) (figure 2). Four alternative CUG codons, 5' to the

AUG codon used for translation of the 155-amino acid form, were used as initiation codons for the high molecular weight forms (Florkiewicz & Sommer, 1989; Arnaud *et al.*, 1999). The different isoforms of the bFGF protein migrate on a SDS-PAGE gel with molecular masses of 18 kDa for the AUG-initiated form and 22, 22.5, 24 and 34 kDa for the CUG-initiated forms (Bikfalvi *et al.*, 1997; Arnaud *et al.*, 1999).

The bFGF proteins initiated at AUG (18 kDa) are primary localized in the cytoplasm, whereas the CUG-initiated forms (22, 22.5 and 24 kDa) are predominantly found in the nucleus (Quarto *et al.*, 1991; Florkiewicz *et al.*, 1991; Bugler *et al.*, 1991). The signal sequence responsible for the nuclear localization of the high molecular forms of

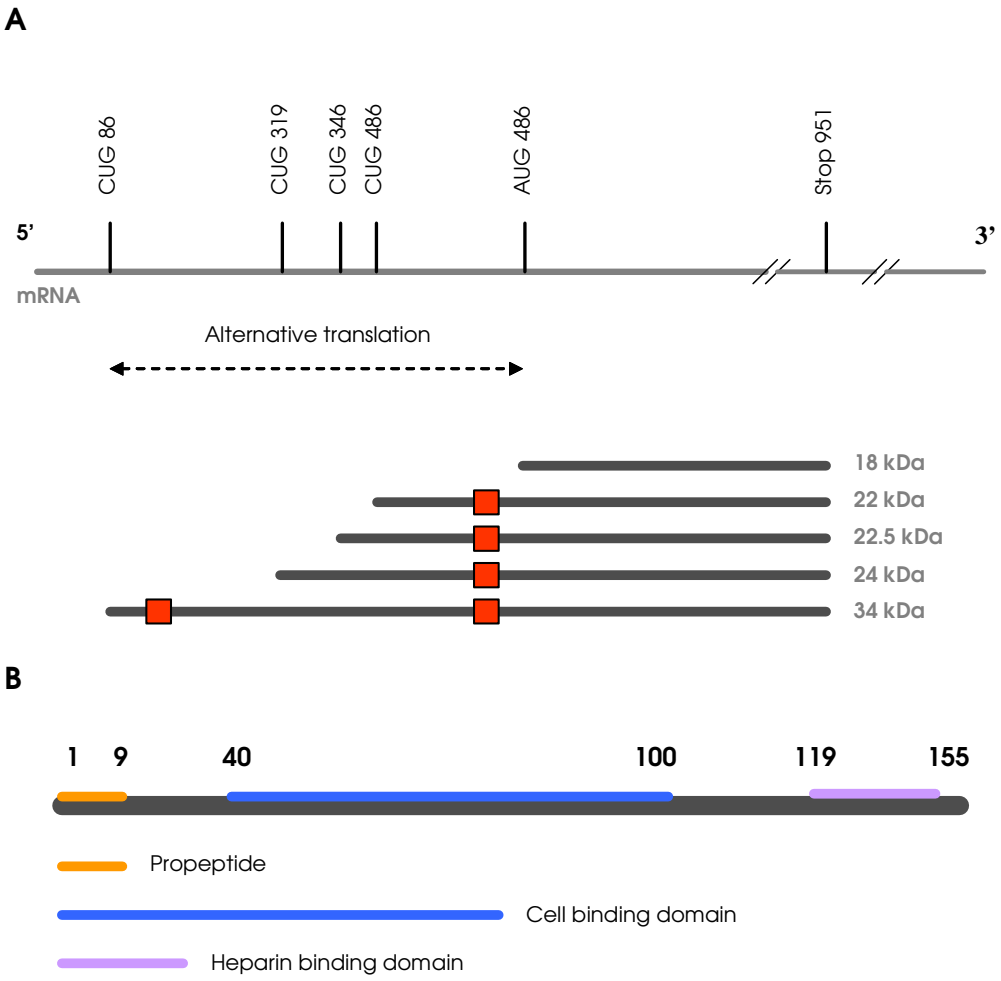


Figure 2: (A) The different isoforms of bFGF as a result of alternative translation. *The upper panel*, shows the human mRNA and codons for translation. In *the lower panel* the bFGF isoforms are shown, 18, 22, 22.5, 24 and 34 kDa-FGF. The red boxes are the nuclear localization signal (NLS). **(B) Structure of the human 18 kDa- bFGF** (155 amino acids). The receptor (cell) binding domain and heparin binding domain are indicated Modified after Okada-Ban *et al.*, 2000.

bFGF derives specifically from a 37 amino acids residue between the last CUG and the AUG start codons within the NH_2-terminal extension (Quarto *et al.*, 1991; Bugler *et al.*, 1991) (figure 2). Furthermore, the 34 kDa bFGF has an additional nuclear localization signal (NLS) between the first and the second CUG start codon (Arnaud *et al.*, 1999) (figure 2). However, nuclear occurrence of the 18 kDa isoform has been reported, suggesting a NLS in the 28 kDa sequence (Claus *et al.*, 2003).

Basic FGF interacts with low-affinity heparin or heparin sulfate proteoglycan (HSPG), which is prerequisite to the biological activity of growth factor (Ornitz & Itoh, 2001). HSPGs are found on cell surfaces and in the extracellular matrix, where their interaction with bFGF stabilizes the growth factor and facilitates its binding to the high-affinity transmembrane FGF receptors (Nugent & Iozzo, 2000; Yayon *et al.*, 1991). Four major FGF receptor families have been identified: FGFR1-4 (Jaye *et al.*, 1992). The FGF receptors consist of an extracellular ligand binding domain, a single transmembrane domain, and an intracellular tyrosine kinase (TK) domain (Ornitz *et al.*, 1996) (figure 1B). The extracellular domain, which consist of three immunoglobulin-like domains (IG loop 1-3), determines the ligand binding specificity and mediates ligand-induced receptor dimerization (Ornitz *et al.*, 1996; Lee *et al.*, 1989). Basic FGF binds to FGFR2 where it has two distinct receptor binding sites, one at the IG loop 2 and another at the interloop region of IG loop 2. Alternative mRNA splicing creates the IIIb and IIIc isoform of the FGFR2, where bFGF only binds to the IIIc isoform (Dow & deVere White, 2000). The bFGF activation of its receptor induces FGFR2 dimerization and subsequently autophosphorylation which initiates signaling cascades.

Basic FGF signaling pathways

After binding to its receptor, bFGF activates a number of intracellular pathways (Nugent & Iozzo, 2000). Here only the most well characterized processes that have been shown to exist in a variety of different cell types will be mentioned (figure 3). Briefly, the proteins FRS2 (FGF receptor substrate 2) and SHC are binding to the phosphotyrosine residue of the bFGF receptor and function as docking protein for the GRB2-SOS complex, which can activate RAS and subsequently initiate the Raf-MEK-MAPK cascade (Pintucci *et al.*, 2002). The MAPK translocates to the nucleus,

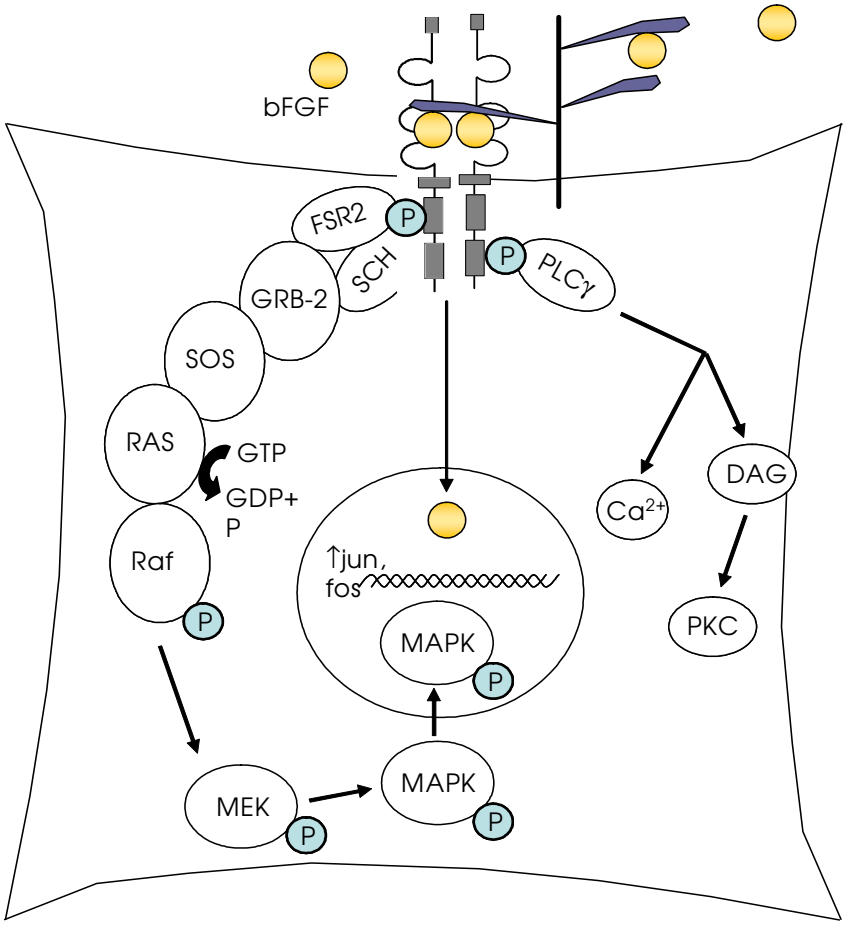


Figure 3: Schematic illustration of bFGF signaling pathway. Basic FGF can activate a number of different intracellular pathways. Here, only the major well characterized processes found in different cell types are shown. Man effector proteins have been left out in order to keep it clear. Modified after Nugent & Iozzo, 2000. See text for explanations.

where it activates transcription factors by phosphorylation. Moreover, phospholipase Cγ (PLCγ) binds also to the phosphotyrosine residue and is activated during bFGF receptor binding. By activation it initiates a signaling cascade via hydrolysis of phosphatidylinositol to inositol-3-phosphate and diacylglycerol (DAG) leading to calcium release and activation of protein kinase C (PKC), which is implicated in a variety of cell functions. Moreover, bFGF has also been shown to be internalized via the cell surface receptors and subsequently translocated and accumulated in the nucleolus, where it is, among others, thought to stimulate the transcription of ribosomal genes during $G_0 \rightarrow G_1$ transition in the cell cycle (Chen *et al.*, 2004).

The binding of bFGF to its receptor as well as internalization of the growth factor is critically dependent upon binding to HSPG, as mentioned earlier, therefore it is

speculated that beside the FGF receptor expressed the type of cellular response is modulated by the specific cell surface HSPG (Nugent & Iozzo, 2000).

Biological function

Basic FGF plays key roles in development, remodeling and disease states in almost every organ system (Bikfalvi *et al.*, 1997) (table 1). The many effects displayed by bFGF indicate the importance of the growth factor in regulation of developmental and physiological activities. Surprisingly, the bFGF knockout mice lacking all isoforms are viable, fertile and are grossly phenotypically similar to the wildtype mice. However, the knockout animals have some mild defects including delayed wound healing, decreased neuronal density in the motor cortex and reduced blood pressure (Dono *et al.*, 1998; Miller *et al.*, 2000; Ortega *et al.*, 1998; Zhou *et al.*, 1998). I will in the following focus on the role of bFGF in the vascular system.

Tab le 1: Function of bFGF in different organ systems. Modified after Bikfalvi *et al., 1997*

Organ	Functions
Blood vessel	**Angiogenesis, smooth muscle cell proliferation, Atherogenesis, blood pressure control**
Brain	Neuronal differentiation and survival
Lung	Branching morphogenesis, fibrosis
Limb	Limb development
Muscle	Myogenesis
Bone	Osseous healing, chondrogenesis
Hematopoiesis	Stimulation of granuloiesis, megakaruocytopoiesis, stem cell survival, anti-apoptotic effect
Reproductive system	Spermatogenesis
Eye	Photoreceptor survival and transduction
Skin	Melanogenesis Morphogenesis of the suprabassal keratinocytes Tissue repair

bFGF in angiogenesis and vascular remodeling

Basic FGF induces migration, proliferation and differentiation of endothelial cells *in vitro*, and hence, it is thought to play an important role in angiogenesis (Dow & deVere White, 2000; Bikfalvi *et al.*, 1997). Furthermore, bFGF has been shown to regulate the expression of several proteins, including interstitial collagenase, urokinase type plasminogen activator (uPa) and β_1 integrins, all of which are important for the invasion of endothelial cells into the matrix during angiogenesis (Bikfalvi *et al.*, 1997). In line with these observations, clinical studies have demonstrated that local as well as systemical injection of bFGF leads to enhanced myocardial collateral flow (Unger *et al.*, 1994; Lazarous *et al.*, 1995; Rajanayagam *et al.*, 2000). Moreover, a recent study was able to show that the bFGF concentration in collateral arteries of patients with chronic total coronary occlusion was higher then in the aortic root, implying a role of bFGF in collateral development and function in those patients (Werner *et al.*, 2004).

Besides bFGF, vascular endothelial growth factor (VEGF) has also been shown to display angiogenetic properties (Ferrara, 2000), and in fact, combined administration of VEGF and bFGF results in greater and more rapid increase of collateral circulation compared with administration of either VEGF or bFGF alone (Asahara *et al.*, 1995). Basic FGF has previously been considered the primary tumor angiogenesis factor (Bikfalvi *et al.*, 1997). This was based upon observations such as elevated bFGF activity in the cerebrospinal fluid of patients with brain tumor and in the urine of patients with solid tumors or leukemias (Bikfalvi *et al.*, 1997).

Basic FGF can induce smooth muscle cell proliferation (Bikfalvi *et al.*, 1997) and has been identified as an important factor in vascular remodeling. Studies suggest that bFGF increases vascular tone (Bryant *et al.*, 1999), a proposal, which is substantiated by the observation that bFGF deficit mice have reduced vascular tone and as a consequence these animals have a low blood pressure (Zhou *et al.*, 1998; Dono *et al.*, 1998). In contrast, Brown *et. al* concluded from experiments done by electric stimulation of muscles in rats that bFGF is a regulator of blood flow by acting as a dilator of small arterioles (Brown *et al.*, 1998) which is supported by the study demonstrating that the diameters of the arterioles in the hamster creek pouch were elevated as a response to bFGF application (Brown *et al.*, 1996). Different setups in

the different studies can be the explanation for the contradictory observations of the implications of bFGF on the vascular system.

bFGF secretion

Basic FGF can display its biological activities by acting as a paracrine (Dow & deVere White, 2000; Seghezzi *et al.*, 1998), an autocrine (Mignatti *et al.*, 1991; Seghezzi *et al.*, 1998) as well as an intracrine factor (Chen *et al.*, 2004). For bFGF to act as an autocrine or a paracrine factor it must be released from the producing cells. However, the striking feature of bFGF is that it lacks a hydrophobic signal sequence, which is needed for secretion via the classical endoplasmatic reticulum-Golgi system (see the following section) (Abraham *et al.*, 1986a; Mignatti *et al.*, 1992; Florkiewicz *et al.*, 1995).

Even though several reports stress the point that cell death or damage might be an explanation for bFGF liberation from cells (Hartnett *et al.*, 1999; Rhoads *et al.*, 2000; McNeil *et al.*, 1989; Brooks *et al.*, 1991) an early study demonstrated that the migration of an isolated single cell expressing bFGF could be inhibited by a bFGF-neutralizing antibody (Mignatti *et al.*, 1991), indicating a mechanism other than cell death or damage leads to bFGF release. This point was further verified by the observations that COS-1 cells selectively secreted the 18-kDa isoform of bFGF and not the high molecular weight (HMW) isoforms (22, 22.5 and 24 kDa), and that the release was dependent upon the α-subunit of the Na^+,K^+ ATPase (Florkiewicz *et al.*, 1995; Florkiewicz *et al.*, 1998). These findings strongly suggest that the bFGF export process is a controlled and active mechanism, distinct from the classical protein secretion. But how is bFGF liberated from the cells? An interesting question, that still is unanswered today although numerous studies have tried to find an answer. In the following, current concepts on protein secretion in general and unconventional protein export in particular will be presented.

Protein secretion

Secretion of proteins via the endoplasmatic reticulum (ER) -Golgi pathway, called the classical or the conventional secretory pathway, is understood in great detail. In

contrast, even though the phenomenon of non-classical protein secretion (also known as un-conventional protein export or ER-Golgi-independent protein secretion) was discovered more than 10 years ago, the molecular mechanisms by which the proteins are exported from the cell are still not clear.

Classical ER-Golgi dependent protein secretion

Almost all proteins synthesised in the cell which are secreted from the cell into the extracellular space need to pass the endoplasmatic reticulum (ER) and the Golgi complex. In mammalian cells, the import of proteins into the lumen of the ER begins before the proteins are fully translated; hence, this import is called a co-translated process. In this process, in which the protein to be secreted is still attached to its ribosome, the protein-ribosome complex will be guided towards the membrane of the ER by a signal sequence at the N'-terminal of the protein. A signal-recognition particle (SRP) leads the protein to the ER membrane, by binding to the signal sequence on the protein (figure 4A). The SRP-ribosome complex binds to a SRP receptor, which is an integral protein exposed only to the cytosolic surface of the ER membrane. This interaction brings the complex to a protein translocator (called Sec61 complex), which is a water-filled pore in the membrane. The SRP and the SRP receptor are then released as the growing polypeptide chain is translocated across the ER membrane through the translocator (figure 4B). The ribosome binds to the translocator by forming a tight seal, insuring that the transferring polypeptide is not lost into the cytosol. The signaling sequence in the polypeptide chain binds to a specific site inside the pore of the translocator, which thereby opens the pore. This means that an ER signaling sequence is recognized twice: first by a SRP in the cytosol and second by a binding site in the ER translocator. The N'-terminal signaling sequence is removed from the polypeptide chain by a signaling peptidase on the luminal side of the ER membrane. However, the signaling sequence remains bound to the translocator while the rest of the protein is transferred through the pore of the membrane as a large loop. When the C-terminus of the protein has passed through the pore, the protein is released into the lumen of the ER and the signaling sequence is released into the ER membrane, where it is rapidly degraded (figure 4C).

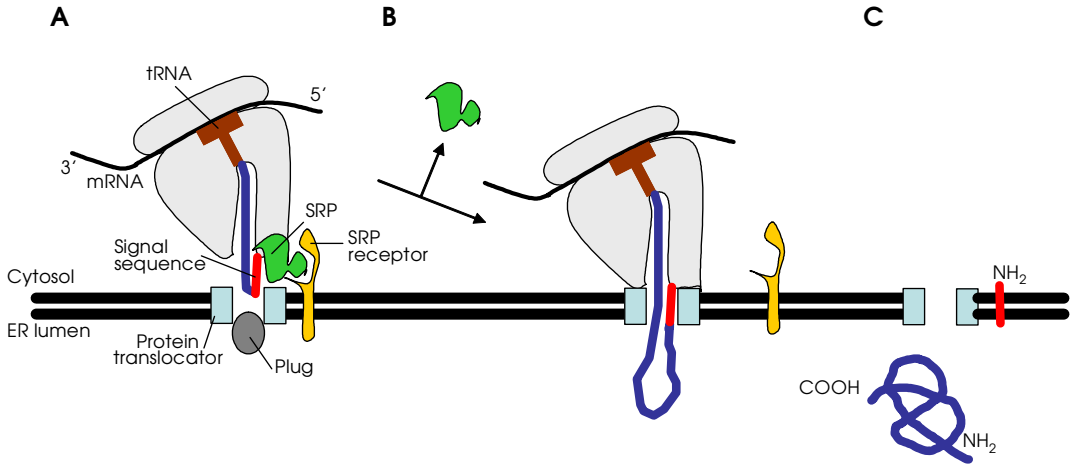


Figure 4: Schematic illustration of the role of the ER signaling sequence in directing secretory proteins into the lumen of ER. In brief, the ER signaling sequence at the NH_2 terminal of the growing polypeptide binds to the SRP (signaling-recognition particle), which guides the growing polypeptide and its ribosome to the membrane of the ER by binding to the SRP-receptor (A). Subsequently, the SRP and its receptor are released enabling the ribosome to bind to the protein translocator. The signaling sequence binds subsequently on the inside of the translocator whereby it is opened making the way for the growing polypeptide to cross the membrane (B). As soon as the C-terminal of the protein crosses the membrane, the signaling sequence is cut of by a signal pepridase and the protein is released into the lumen of the ER, while the signaling sequence is left in the membrane for rapid degradation (C). Modified after Alberts *et al.*, 2002b.

When the protein is inside the ER it is glycosylated, which is thought to be prerequisite for proper folding of the protein. The folding is necessary for a protein to leave the ER. The proteins destined for secretion are leaving the ER and enter the Golgi apparatus. Proteins coming from the ER enter the cis Golgi network, are then undergoing a series of covalent modifications followed by exit from the trans Golgi network for their destination. In the trans Golgi network, the proteins are sorted in three classes: Those destined for lysosomes, those for immediate delivery to the cell surface and those for secretory vesicles. In the last case, the secretory vesicles containing the proteins to be secreted are bud of the trans Golgi network and transported to the cell membrane with which the vesicles fuse and release their proteins by exocytosis. Release of proteins from cells can occur continuously (termed constitutive secretion) or the secretory proteins can be stored intracellularly in specialized secretory granules for release upon an appropriate stimulus (regulated secretion). Reviewed in Alberts *et al.*, 2002c and Alberts *et al.*, 2002b.

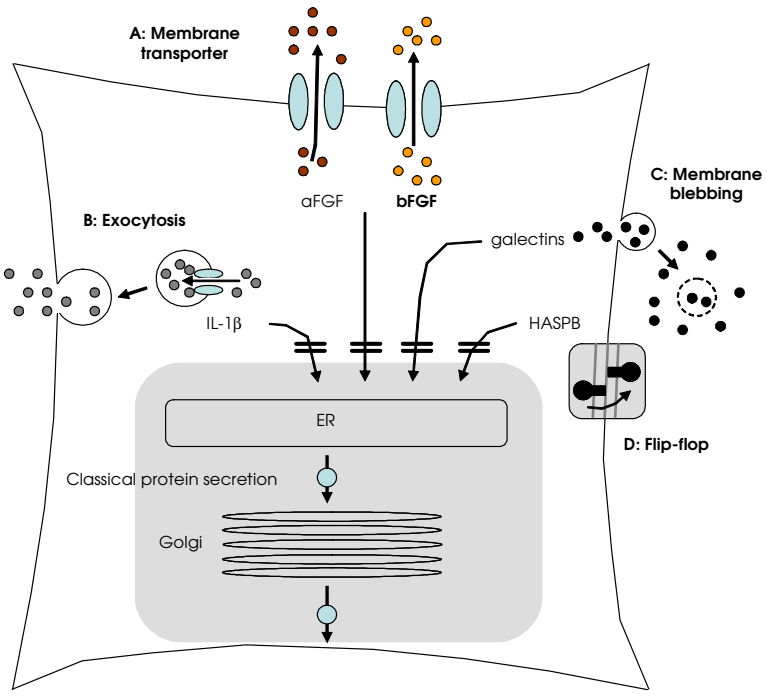


Figure 5: Schematic illustration of potential export routes of non-classical protein secretion. Four distinct types are shown here: Direct translocation across the cell membrane (A), exocytose, where vesicles containing proteins are fused with the membrane whereby the proteins are released (B), membrane blebbing, where small parts of the membrane are bud of enclosing proteins (C), and a flip-flop mechanism directly translocating membrane proteins across the membrane (D). Modified after Nickel, 2003.

Non-classical ER-Golgi independent protein secretion

Basic FGF is just one protein among a large group of proteins which are secreted from the cells where they are synthesised in a non-classical way. The group of unconventional secreted proteins includes FGF-1 (aFGF) (Jackson *et al.*, 1995; Jackson *et al.*, 1992), interleukin-1β (IL-1β) (Rubartelli *et al.*, 1990; Andrei *et al.*, 1999) and galectins (Hughes, 1999) and at least four kinds of mechanistically distinct non-classical export routes have been suggested (Hughes, 1999; Nickel, 2003) (figure 5), which briefly will be reviewed here.

Direct translocation from the cytosol to the extracellular space across the plasma membrane is one way for proteins to be secreted from cells (Nickel, 2003) (figure 5A). Basic FGF and aFGF have been suggested to be secreted in this way (Engling *et al.*, 2002; Backhaus *et al.*, 2004).

Exocytosis is another way by which proteins can be liberated from cells (Nickel, 2003). In this way proteins are kept in vesicles which are fusing with the cell membrane and thereby secrete the protein to the extracellular space (figure 5B). It is thought that IL-1β is exported from the cells in this way (Andrei *et al.*, 1999). An ATP-binding-cassette (ABC)-transporter, ABC1, is a protein which is implicated in the transport of proteins from the cytosol to the plasma membrane and it is suggested to

mediate the translocation of IL-1β from the cytosol to the lumen of the vesicles (Hamon *et al.*, 1997).

Membrane blebbing is a third non-classical protein secretion pathway (Nickel, 2003). In this way proteins are accumulated directly at the cytosolic side of the cell membrane, followed by formation of membrane-bound vesicles (also called exosomes) that pinch off the membrane before the proteins are being released into the extracellular space (figure 5C). Members of the galectin protein family are also thought to be secreted in this way (Hughes, 1999).

A flip-flop mechanism has been suggested for translocation of membrane – anchored proteins across the plasma membrane (figure 5D). A protein such as the cell surface protein called HASPB (hydrophilic acylated surface protein B) is proposed to be released in this way (Nickel, 2003).

Stimuli for bFGF secretion

Whereas only little is known about the mechanism behind the release of bFGF from the producing cells, different stimuli causing the liberation of the growth factor without disrupting the cell membrane have been identified: Estrogen has been shown to be able to induce release of bFGF (Albuquerque *et al.*, 1998), presumably by cross-talking with TGF-β3 (Chaturvedi & Sarkar, 2004; Hentges & Sarkar, 2001). Furthermore, hypoxia (Ishibashi *et al.*, 2001; Ishibashi *et al.*, 1995; Ambalavanan *et al.*, 1999; Kuwabara *et al.*, 1995) and inflammatory cytokines such as tumor necrosis factor-α, interferon-γ, interleukin-1β (Samaniego *et al.*, 1995; Samaniego *et al.*, 1997) as well as interferon-α and interleukin-2 when they are administrated in combination (Cozzolino *et al.*, 1993) stimulate cells to release bFGF. Additionally, stimuli such as nicotine (Cucina *et al.*, 1999b), thrombine (Cucina *et al.*, 1999a; Herbert *et al.*, 1994) and elevated hydrostatic pressure (Acevedo *et al.*, 1993) are also influencing the release of the growth factor.

Interestingly, shear stress has been demonstrated to be a very potent stimulus for bFGF release from endothelial cells *in vitro* (Gloe *et al.*, 2002) as well as *in vivo* (Lepidi *et al.*, 1995). However, the knowledge about how the shear stress as a mechanical signal is translated into the biochemical response represented by a release of bFGF is only very limited. The study from Gloe *et al.* (2002) showed that the shear stress

induced bFGF release was critical dependent upon integrin $\alpha_v\beta_3$, indicating that this integrin is involved in the mechanotransduction process. It is, however, not clear in what way this integrin is contributing to the bFGF release.

Shear stress and signaling transduction

Shear stress is the mechanical force exerted on endothelial cells, which are lining the inner surface of the vessels, when blood is flowing in the vessels. An increase in blood flow velocity through a vessel, as a result of for example exercise, elevates shear stress on the endothelial surface. As an early response to shear stress, the vessels will dilate, to be able to deliver more blood to the working muscles in order to meet their increased demand of oxygen and nutrients. By chronic shear stress, for example as a consequence of training, enlargement of existing vessels, arteriogenesis, as wells as formation of new capillaries, angiogenesis, will occur.

More than 150 years ago, Virchow observed the ability of the vascular endothelium to sense and respond to the flow of blood, referring to the heterogeneous morphology of the endothelium along the arterial tree, which correlated with the patterns of flow to which the cells were exposed (Resnick *et al.*, 2003). Since then, a substantial amount of studies have investigated the response of the endothelium to changes in blood flow. The endothelium can react to an acute increase in blood

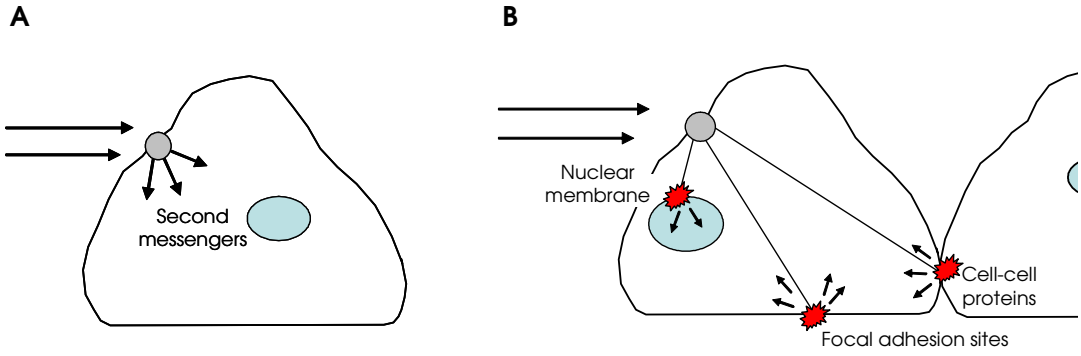


Figure 6: Illustration of two signaling transduction models. (A) The shear stress force is sensed by a mechanosensor located on the luminal cell surface. This mechanosensor can be a cell surface protein or mechanoactivated ion-channels. The signal is subsequently submitted within the cell via second messengers. (B) The shear stress force on the luminal surface is recognized by a membrane protein, which can transmit the signal by cytoskeletal deformation. The stress stimuli are subsequently transmitted to site remote from the origin, where the cytoskeleton is connected to membrane proteins (marked in red), such as intracellular junctions (e.g. PECAM), abluminal focal adhesion attachment (e.g. integrins) and nuclear membrane. Modified after Davies, 1995.

flow by dilatation of arteries through nitric oxide (NO), prostaglandins, and hyperpolarizing factors (Frangos *et al.*, 1985; Pohl *et al.*, 1986; Huang *et al.*, 2001), as well as to chronic flow alterations by structural remodeling of the artery wall (Langille & O'Donnell, 1986). But how does the endothelium sense the shear stress and translate it into biochemical responses?

It has been suggested that structures on the surface of endothelial cells function as a flow sensors (Mazzag *et al.*, 2003). Such sensors are not only located on the luminal surface of the endothelium but may also be located at intercellular connections (PECAM) or abluminal side (integrins) being activated directly by mechanical deformation of the cell or indirectly by altered load of the cytoskeleton (Mazzag *et al.*, 2003; Berk *et al.*, 1995; Davies *et al.*, 1997; Davies, 1995) (figure 6). However, a substantial amount of evidence points to that integrins are directly involved in the signaling transduction in endothelial cells under shear stress (Tzima *et al.*, 2001; Pampori *et al.*, 1999; Davies, 1995). This idea is further substantiated by the fact that many of the signaling pathways activated by integrin stimulation are also activated by shear stress (Shyy & Chien, 2002; Shyy & Chien, 1997). However, the knowledge about how integrins directly can be activated during shear stress is still not complete.

Integrins and cell-matrix interaction

Integrins are the sites primary responsible for cell adhesion and are involved in the regulation of endothelial cell signaling, morphology, proliferation, migration and differentiation (Davies, 1995). They are membrane-associated glycoproteins composed of α and β subunits. Each subunit has a large extracellular domain, a transmembrane spanning region and a short cytoplasmatic domain. The extracellular domain binds directly to the extracellular matrix (ECM) proteins, such as vitronectin, fibronectin, laminin and collagen (Shyy & Chien, 2002). The cytoplasmatic domain is associated with adapter proteins such as the cytoskeletal proteins α-actinin, vinculin, talin, tensin and paxillin, and the signaling molecules focal adhesion kinase (FAK), Src family protein tyrosine kinases (PTK's) and p130Cas (termed focal adhesion), all of which are tyrosine phosphorylated by integrin-

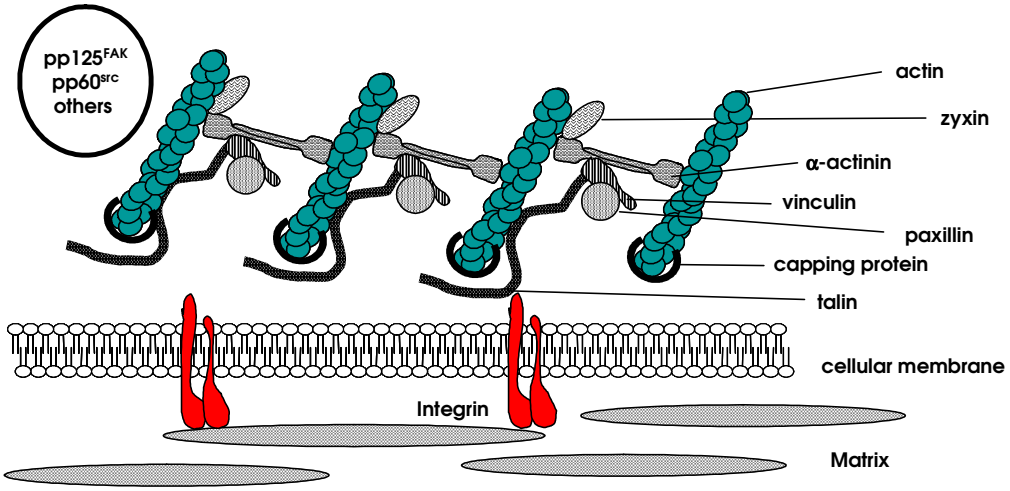


Figure 7: Schematic illustration of the proteins involved in the focal adhesion site. Modified after Gloe & Pohl, 2002.

mediated cell adhesion to the ECM (Shyy & Chien, 2002;Shyy & Chien, 1997) (figure 7).

The focal adhesions are perhaps the most intensively studied cytoskeletal-linked mechanotransductions sites. The first evidence that integrins are implicated in mechanotransduction came from the observation that shear stress applied to the luminal surface of endothelial cells resulted in a directional remodeling of the abluminal focal adhesion sites (Davies *et al.*, 1994; Davies *et al.*, 1993). As integrins bind to the ECM, they become clustered in the plane of the cell membrane and via the associated adapter proteins they can initiate signaling transduction (Giancotti & Ruoslahti, 1999; Shyy & Chien, 2002). However, even in quiescent cells there is a rapid turnover of integrin attachment and detachement (Davies, 1995) and hence, it is not fully understood what the signal for integrin activation and subsequently intracellular signaling is. However, proteases have been shown to influence integrins positively and thereby contribute to their activation (Preston *et al.*, 2002; Geiger *et al.*, 2001; Mott & Werb, 2004; Faisal Khan *et al.*, 2002; Koshikawa *et al.*, 2000), but it is still unknown whether proteases are able to mediate integrin activation in response to shear stress.

Intracellular signaling

As mentioned previously, endothelial cells can respond in an acute and a chronic way to enhanced shear stress (Berk *et al.*, 1995; Tseng *et al.*, 1995). It has been suggested that the acute and transient response is mediated by a calcium dependent pathway, whereas the slow and sustained signal is mediated by a calcium-independent pathway. The calcium dependent pathway involves activation of phospholipase C, hydrolysis of phosphatidylionsitol 4,5-biphosphate (PIP2), increase in intracellular calcium and formation of the calcium-calmodulin complex and stimulation of PKC, leading to activation of nitric oxide synthase (NOS) and ion transport (figure 8B). In contrast, the calcium-independent pathway involves activation of a small GTP-binding protein and stimulation of calcium-independent PKC and MAP kinases, leading to a sustained activation of NOS and changes in cell

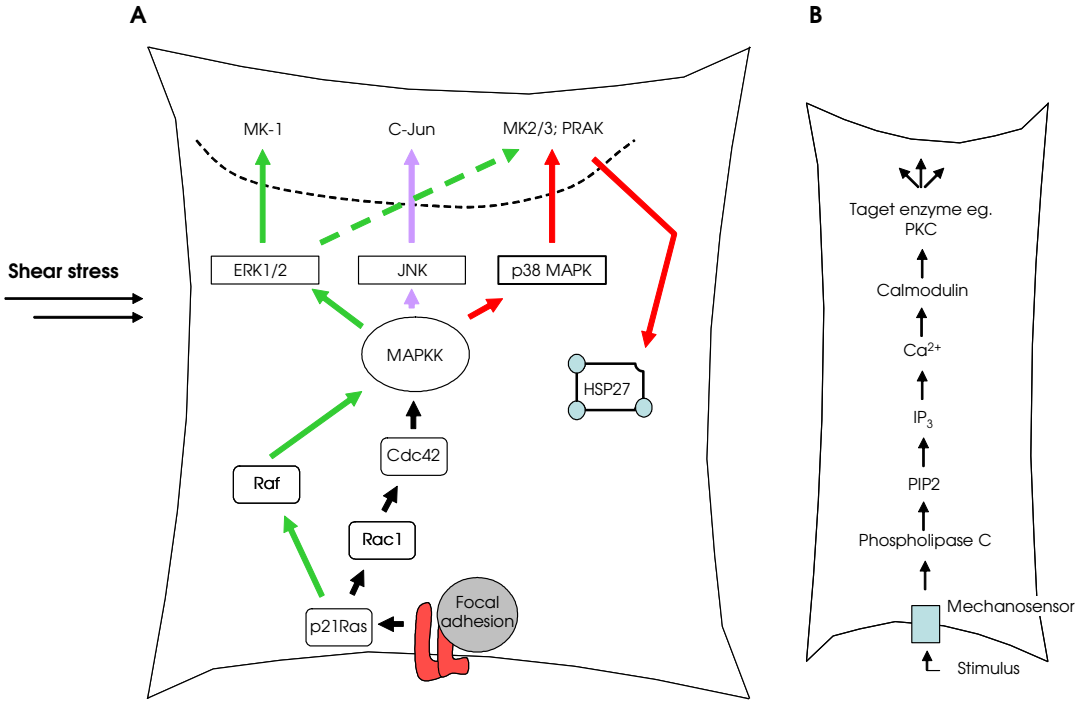


Figure 8: Schematic diagram of the shear stress-induced pathways. (A) The calcium-independent pathway involving integrin-mediated MAP kinase activation. The p38 MAPK pathway can induce heat shock protein 27 (HSP27) phosphorylation via MK (MAPKAP kinase) 2/3 and PRAK (MAPKAP kinase 5. ERK1/2 (p42/44 MAP kinase) can also activate MK2 and PRAK and thereby presumably phosphorylate HSP27 (see text for more information). The thin black dotted line indicates nucleus. **(B)** The calcium-dependent pathway activated by shear stress. Modified after (Berk *et al.*, 1995) and Tseng *et al.*, 1995.

morphology and gene expression (Berk *et al.*, 1995; Tseng *et al.*, 1995).

For a long time it has been known that shear stress can activate the extracellular signaling-regulated kinase (ERK1/2) pathway as well as the c-Jun NH_2-terminal protein kinase (JNK) pathway (Azuma *et al.*, 2000; Takahashi & Berk, 1996; Minden *et al.*, 1995; Coso *et al.*, 1995; Tseng *et al.*, 1995; Jo *et al.*, 1997). The small GTPases p21Ras, Rac1 and Cdc42 are activated during shear stress in an integrin-dependent manner initiating the JNK pathway leading to c-Jun transcription (Coso *et al.*, 1995; Minden *et al.*, 1995; Tzima *et al.*, 2003; Tzima *et al.*, 2002). On the other hand, the shear stress induced integrin-dependent Ras and Raf activation seems to be implicated in the activation of the ERK1/2 pathway (Shyy & Chien, 2002; Li *et al.*, 1999; Jo *et al.*, 1997) (figure 8A). Recently, Azuma *et. al* (2001) demonstrated that shear stress not only induces activation of ERK1/2 and JNK but also the p38 MAPK-MAPKAPK-II pathway resulting in phosphorylation of heat shock protein 27 (HSP27) (figure 8A), a pathway which has been implicated in cell shape changes and migration (Piotrowicz *et al.*, 1998; Hedges *et al.*, 1999; Rousseau *et al.*, 1997). Since shear stress is able to induce release of bFGF from cells, as mentioned earlier, it may be hypothesized that the phosphorylation of HSP27 plays a role in the release of bFGF during shear stress.

The p38 MAPK pathway and HSP27 phosphorylation

Like the other MAP kinases, p38 MAP kinase is predominantly localized in the cytoplasm (Seternes *et al.*, 2002). However, upon environmental or cellular stress, p38 MAPK is activated by phosphorylation, which leads to a translocation of the p38 MAPK to the nucleus (Chen *et al.*, 1992). *In vitro* as well as *in vivo,* the p38 is the activator for MAPKAP kinase-2 (MK2) (Rouse *et al.*, 1994), MAPKAP kinase-3 (MK3) (McLaughlin *et al.*, 1996), and MAPKAP kinase-5 (PRAK (p38 regulated and – activated protein kinase)) (Ni *et al.*, 1998; New *et al.*, 1998), which in the inactivated forms are localized in the nucleus (Seternes *et al.*, 2002; Lenormand *et al.*, 1993) (figure 8A). Following phosphorylation of MAPKAP kinases, nuclear p38 MAPK is exported to the cytoplasm in a complex with the given MAPKAP kinase (Engel *et al.*, 1998; Ben Levy *et al.*, 1998; Seternes *et al.*, 2002). In the cytoplasm, the MAPKAP kinase 2/3 and PRAK phosphorylate HSP27 (Stokoe *et al.*, 1992b; Clifton *et al.*, 1996; Ni

et al., 1998; New *et al.*, 1998). *In vitro*, the p42/44 MAPK has been shown also to be able to activate MAPKAP kinase-2 (Clifton *et al.*, 1996; Stokoe *et al.*, 1992a) and thereby HSP27 (Huot *et al.*, 1995). However, other studies could not show the HSP27 phosphorylation via the p42/44 pathway (Guay *et al.*, 1997; Rouse *et al.*, 1994; Gaitanaki *et al.*, 2003). Moreover, p42/44 MAPK is also able to activate PRAK and by that phosphorylate HSP27 (Ni *et al.*, 1998).

HSP27 is a stable protein present at low levels in nearly all cells and tissues, and is a member of a large family of chaperones, which assists in the folding of many different proteins (Alberts *et al.*, 2002a). In unstressed cells, HSP27 is found in the cytosol predominantly as a large oligomeric unit of 500 – 800 kDa, usually consistent of six tetrameric complexes of the protein (Arrigo & Welch, 1987; Arrigo *et al.*, 1988). An enhanced expression and phosphorylation of HSP27 is seen as a response to estrogen (Piotrowicz *et al.*, 1995), different forms of stress, such as heat shock (Rouse *et al.*, 1994; Landry *et al.*, 1992; Arrigo *et al.*, 1988), oxidative stress (Barchowsky *et al.*, 1994) as well as shear stress (Piotrowicz *et al.*, 1997; Azuma *et al.*, 2001; Li *et al.*, 1996), and cytokines such as interleukin 1 (IL-1) (Freshney *et al.*, 1994; Guesdon *et al.*, 1993), TNF (Landry *et al.*, 1992; Guesdon *et al.*, 1993), thrombin and histamine (Levin & Santell, 1991), many of which also leads to an active release of bFGF, as mentioned earlier.

Phosphorylation occurs on three different serine residues, Ser-15, Ser-78 and Ser-82 (Landry *et al.*, 1992) resulting in a re-distribution of the large oligomer into smaller tetrameric units (Kato *et al.*, 1994) and translocation to the nucleus (Arrigo & Welch, 1987; Arrigo *et al.*, 1988). *In vitro*, HSP27 behaves as an actin-capping protein, which in the non-phosphorylated form inhibits actin polymerization, whereas when HSP27 is phosphorylated it seems to leave the actin, allowing the actin to polymerize (Lavoie *et al.*, 1995; Guay *et al.*, 1997; Benndorf *et al.*, 1994; Miron *et al.*, 1991; Miron *et al.*, 1988). The dynamics of the microfilament polymerization is important for lamellopodia formation and morphology observed during shear stress, and thus for the motility of endothelial cells (Piotrowicz *et al.*, 1998) and smooth muscle cells (Hedges *et al.*, 1999). Furthermore, transfection of bovine aortic endothelial cells (BAEC) with human HSP27 resulted in a 2-3 fold enhancement cellular growth (Piotrowicz *et al.*, 1995) and additionally it has been demonstrated that phosphorylation of HSP27 is required for cell migration (Piotrowicz *et al.*, 1998), two

processes which are also mediated by bFGF (Nugent & Iozzo, 2000). Moreover, HSP27 has interestingly been shown to co-precipitate with bFGF and has therefore been proposed to facilitate the release of the growth factor (Piotrowicz *et al.*, 1997). However, it is not known how HSP27 can facilitates the bFGF release, but it is suggested that the function of HSP27 is activated by phosphorylation (Guay *et al.*, 1997), giving rise to the hypothesis that activated HSP27 could play an important role in the release of bFGF during shear stess.

Hypothesis

In the view of the fact that shear stress induces an integrin-dependent release of bFGF from endothelial cells, it is in this thesis hypothesized that the shear stress induced bFGF liberation from endothelial cells is controlled by cell-matrix interactions and since proteases are able to remodel the extracellular matrix it is further hypothesized that proteases are required for the activation of integrin $\alpha_v\beta_3$ and subsequently initiation of the intracellular signaling cascade resulting in the bFGF release.

Moreover, since shear stress induces HSP27 phosphorylation, it is hypothesized that HSP27 in the phosphorylated form acts as a carrier for bFGF upon its release through the cell membrane.

Aims

In view of the importance of bFGF in biological systems it seems essential to gain more information about its release mechanism. Whereas shear stress is a known stimulus for bFGF release from endothelial cells the chain of molecular mechanisms by which the shear stress forces are translated finally into release of bFGF still needs to be elucidated. As pointed out in the introduction, numbers of studies have looked at integrins as possible mechanotransducers, and recently, proteases have been suggested as potential integrin activators. Hence, the first aim of the present study is to clarify the role of proteases in the mechanotransduction of shear stress forces into

the release of bFGF from endothelial cells. A second aim of this study is to elucidate the role of HSP27 in facilitating the release of bFGF.
In detail, the following questions were aimed to be answered in this context:

1. Mechanotransduction of shear stress into bFGF release:
   - Do cells have to be attached to the extracellular matrix in order to release bFGF in respond to shear stress?
   - Do proteases play a role in the shear stress-induced bFGF release by remodeling the extracellular matrix?
     - Is protease activity increased during shear stress?
     - What kind of protease could be involved?
     - Can proteases, when administrated alone, induce bFGF release in static cells?
     - Do protease treatment and shear stress induce similar pathways in endothelial cells?
   - How can the integrin $\alpha_v\beta_3$ contribute to the bFGF release?

2. bFGF release:
   - Which intracellular signaling cascade is involved
   - Does HSP27 phosphorylation play a role?
   - Are HSP27 and bFGF interacting?
   - How is bFGF released?

Materials and methods

Materials

All **cell culture reagents** were obtained in highest purity from Sigma (Taufkirchen, Germany), except from **fetal calf serum** which was purchased from Biomol (Hamburg, Germany). The **L-15 medium** used in shear stress experiments performed with the parallel plate system is also obtained from Sigma (Taufkirchen, Germany). All the **membranes** used for sterilization was purchased from MembraPure GmbH (Bodenheim, Germany). **Laminin type I (mouse)** used for coating the culture dishes was from Harbo Bio-Products (Norwood, MA, USA) and the **collagen type I** was purchased from Genaxxon bioscience (Stafflangen, Germany). **Porcine pancreatic elastase** utilized for treating static cells with elastase was obtained from SERVA electrophoresis (Heidelberg, Germany) and the **elastase specific substrate** (MeOSuc-Ala-Ala-Pro-Val-pNA) from Bachem (Weil am Rhein, Germany). The **bFGF ELISA kit** was obtained from R&D systems (Wiesbaden-Nordenstadt, Germany). The **enhanced chemoluminescence protein detection kit** was from Applichem (Darmstadt, Germany) and the **BCA protein assay kit** was purchased from Pierce (Bonn, Germany). 3,3',5,5' tetramethylbenzidine (**TMB**) was obtained from Sigma (Taufkirchen, Germany). The **dialyzing tubes** were from MembraPure GmbH (Bodenheim, Germany) and the **ampholytes** pH 3-10 as well as pH 5-8 was purchased from Fluka (Taufkirchen, Germany). The **protein A- micro beads** and the **MACS columns** utilized for immuno-precipitation were obtained from Miltenyi Biotec. (Auburn, CA). The **CellWASH** and the **propidium iodid** used in FACS analysis were from BD Biosciences (Erembodegem, Belgium). The **polyacrylamid:bis solution** for making SDS-PAGE was obtained from Genaxxon bioscience (Stafflangen, Germany). The **nitrocellulose membrane** used for Western blot was from PeqLab (Erlangen, Germany). **Formaldehyde** used for fixation of cells was obtained from Sigma (Taufkirchen, Germany). **Silicone solution®** was obtained from SERVA electrophoresis (Heidelberg, Germany).

Media

DMEM (10%)

Dulbecco's modified Eagle's medium (DMEM) (Dulbecco & Freeman, 1959)	445 ml
Penicillin (10000 U) / streptomycin (10 mg)	2 ml
L-Glutamine (200 nM)	5 ml
Fetal calf serum (10%)	50 ml
pH 7.4	

DMEM (1%)

Dulbecco's modified Eagle's medium (DMEM) (Dulbecco & Freeman, 1959)	450 ml
Penicillin / streptomycin	3 ml
Glutamine (200 nM)	5 ml
Fetal calf serum (1%)	4.5 ml
pH 7.4	

Leibovitz L-15 medium

Leibovitz L-15 medium (LEIBOVITZ, 1963)	445 ml
Penicillin (10000 U) / streptomycin (10 mg)	2 ml
L-Glutamine (200 nM)	5 ml
Fetal calf serum (1%)	4.5 ml
pH 7.4	

Sterilization of all the media was done by passing the media through two filters. First a Glasfaser filter (C5) with a pore size of 0.45 µm and second a Memfil CA with a pore size of 0.2 µm.

Buffers and solutions

Anolyte

Phosphoric acid (85%) 0.68 ml

Distilled H_2O 1000 ml

Blocking buffers

A-blocking buffer (3% milk)

3% non-fat dry milk in TBST

B-blocking buffer (5% BSA)

5% BSA in TBST

C-blocking buffer (5% BSA)

5% BSA in PBS(-)

D-blocking buffer (1% BSA)

1% BSA in PBS(-)

E-blocking buffer (10% FCS)

10% FCS in PBS/Triton

F-blocking buffer (10% FCS)

10% FCS in PBS(-)

Catholyte

NaOH 2.4 g

Distilled H_2O 600 ml

Freshly made and degassed immediately prior to use

Citrate buffer (15 mM)

135 mM KCl		10.06 g
15 mM NaCitrate		4.4 g
H_2O	ad	1000 ml

Collagenase solution (170 U/ml)

Collagenase type I (1700 U/mg)	1 g
PBS(+)	1000 ml

The collagase solution was passed through the filter Membrex 25 CA with a pore size of 0.2 µm in order to sterilize the solution.

Elastase (0.5 U/ml)

Porcine pancreatic elastase (130 U/ml)	7.6 µl
DMEM (1%)	2 ml

Formaldehyde

3.7% formaldehyde

3.7% formaldehyde in PBS(-)

4% formaldehyde

4% formaldehyde in PBS(-)

10% formaldehyde

10% formaldehyde in PBS(-)

H_2O_2 (0.6%)

0.6% H_2O_2 (30% solution) in PBS/Triton.

Always freshly made prior to use.

IEF-gel (13.5%):

Urea	16.3 g
10% Triton-X-100	6 ml
Distilled H_2O	6 ml
Acrylamide/Bisacrylamide 30%/0.8% (w/v)	4.05 ml
Ampholyte pH 5-8	1.2 ml
Ampholyte pH 3-10	0.3 ml
APS	0.06 ml
TEMED	0.03 ml

IEF solubilization:

Urea		5.7 g
10% triton-X-100		2 ml
Ampholyte pH 3-10		0.2 ml
Mercaptoethanol		0.5 ml
H_2O	ad	10 ml

IEF-washing buffer:

Tris-HCl (pH 8.0)		1.2 g
Methanol		1000 ml
SDS		20 g
H_2O	ad	2000 ml

Laminin (10 µg/ml)

Laminin type I (2.5 mg/ml)	4 ml
PBS(+)	996 ml

The laminin solution was passed through the filter Membrex 25 CA with a pore size of 0.2 µm in order to sterilize the solution.

Lysis buffer (w. Triton-X-100):

KH_2PO_4	20 mM
EDTA	1 mM
Pefablock	1 mM
Leupeptin	1 µM
Pepstatin	1 µM
NaF	50 mM
Na_4-pyrophosphate	40 mM
Na_3VO_4	2 mM
Triton-X-100	1 %

pH = 7.4

Lysis buffer (w/o Triton-X-100):

KH_2PO_4	20 mM
EDTA	1 mM
Pefablock	1 mM
Leupeptin	1 µM
Pepstatin	1 µM
NaF	50 mM
Na_4-pyrophosphate	40 mM
Na_3VO_4	2 mM

pH = 7.4

Overlay buffer:

IEF solubilization buffer diluted 1:3 with water with Bromophenol blue

Phosphate buffered saline (PBS)

PBS (-):

NaCl		8.00 g
KCl		0.20 g
$Na_2HPO_4x\ 2H_2O$		1.42 g
KH_2PO_4		0.20 g
H_2O	ad	1000 ml
pH 7.4		

PBS(+):

NaCl		0.80 g
KCl		0.20 g
Na_2HPO_4		1.15 g
KH_2PO_4		0.20 g
$MgCl_2$		0.04 g
H_2O	ad	1000 ml

PBS(-) was autoclaved and PBS(+) was passed through the filter Memfil CME with a pore size of 0.2 µm in order to sterilize the solutions.

PBS/Triton

PBS(-) containing 0.1% Triton-X-100

Running buffer (5x):

Tris Base		75 g
Glycine		360 g
SDS		25 g
H_2O	ad	5 l

Sample / loading buffer (4x):

Tris-HCl (pH 6.8)	0.25 M
SDS	8 %
Glycerine	40 %
Bromphenolblue	0.02 %
Mercaptoethanol	400 mM

SDS-PAGE:

Separations gel:

	10%	**12%**
H_2O	20.00 ml	16.7 ml
Tris (1.5 M) (pH 8.8)	12.50 ml	12.5 ml
SDS 10% (w/v)	0.50 ml	0.5 ml
Acrylamide/Bisacrylamide 30%/0.8% (w/v)	16.60 ml	20.0 ml
Ammoniumpersulfate (APS) 10% (w/v)	0.25 ml	0.25 ml
TEMED	0.025 ml	0.025 ml

Stacking gel (4%):

H_2O	6.00 ml
Tris (0.5 M) (pH 8.8)	2.50 ml
SDS 10% (w/v)	0.10 ml
Acrylamide/Bisacrylamide 30%/0.8% (w/v)	1.30 ml
Ammoniumpersulfate (APS) 10% (w/v)	0.05 ml
TEMED	0.01 ml

Sodium orthovanadate (Na_3VO_4) (100mM)

Na_3VO_4	183,9 mg
dH_2O	10 ml

The sodium orthovanadate should be activated for maximal inhibition of tyrosine phosphotases. By the following activations procedure the vanadate was depolymerized:

1. The pH of Na_3VO_4 solution was adjusted to pH 10.0. At this pH the solution is yellow
2. The solution was boiled until it turned colorless (approximately 10 min)
3. It was cooled down to room temperature
4. The pH was readjusted to pH 10.0 and the steps 2 and 3 was repeated until the solution remained colorless and the Ph stabilized at 10.0

TBST:

Tris-HCl (pH 7.4)	50 mM
NaCl	150 mM
Tween 20	0.3 %

Transfer buffer:

Clycine	39 mM
Tris Base	48 mM
SDS	0.037 %
MeOH	10 %

Antibodies

Primary antibodies

The primary antibodies used in the study are listed in table 2.

Table 2: Primary antibodies. PC = Polyclonal antibody; MC = Monoclonal antibody; FA = Fractionated antiserum.

Antibody	Raised in	Company	Place	Product #	Application	Conc./ dilution
Anti-HSP27	Rabbit (PC)	Stressgen	Hamburg, DE	SPA-803	Western Blot	1:5000
					Immuno-staining	1:200
					Modified ELISA	20 µg/ml
	Mouse (MC)	Acris	Bad Neuheim, DE	BM152	Isoelectric focusing	1:1000
Anti-phospho HSP27 (Ser-78)	Mouse (MC)	Upstate	Hamburg, DE	05-645	Modified ELISA	20 µg/ml
Anti-phospho HSP27 (Ser-82)	Rabbit (PC)	Cell Signaling	Frankfurt, DE	2401	PACE	1:200
Anti-bFGF	Rabbit (FA)	Sigma	Taufkirchen, DE	F3393	FACS	1:200
					FACE	1:400
	Mouse (MC)	R&D systems	Wiesbaden, DE	CONJUGATE08	Modified ELISA	50 µl

Anti-p38	Rabbit (PC)	Stressgen	Hamburg, DE	KAS-MA009	Western Blot	1:5000
Anti-phospho p38 (Thr180/Tyr182)	Mouse (MC)	Cell Signaling	Hamburg, DE	28B10	Western Blot	1:2000
Anti-phospho p42/44 (Thr202/Tyr204)	Mouse (MC)	Cell Signaling	Hamburg, DE	9106S	Western Blot	1:500
Anti-phospho serine	Rabbit (PC)	Abcam	Cambridge,UK	Ab9332	Immuno-precipitation	0.25µg /100µg protein
Anti-actin	Goat	Santa Cruz	Heidelberg, DE	I-19	Western blot	1:500
Anti-PECAM	Goat	Santa Cruz	Heidelberg, DE	M-20	Western blot	1:500

Secondary antibodies

Table 3 gives an overview of the horseradish peroxidase (HRP)-conjugated secondary antibodies used in this study.

Table 3: HRP-conjugated secondary antibodies

Antibody	Raised in	Company	Place	Product #	Application	Dilution
Anti-mouse	Goat	Calbiochem	Schwalbach, DE	DC-08L	Western blot	1:25000
Anti-rabbit	Goat	Calbiochem	Schwalbach, DE	DC-03L	Western blot PACE FACE Self-made ELISA	1:25000 1:400 1:800 1:400
Anti-goat	Rabbit	Calbiochem	Schwalbach, DE	DC-03L	Western blot	1:10000

In table 4 are shown the fluoreceine-isothiocyanate (FITC)-labled secondary antibodies which were utilized for immunohisto-chemistry and FACS analysis.

Table 4: Fluorescent labeled secondary antibody

Antibody	Raised in	Company	Place	Product #	Application	Dilution
Anti-rabbit	Goat	Invitrogen	Karlsruhe, DE	65-6111	Immuno-staining	1:400
Anti-rabbit	Chicken	Invitrogen	Karlsruhe, DE	A-21441	FACS	1:400

Inhibitors

A list of inhibitors used in the study is found in table 5.

Table 5: Overview of used inhibitors.

Inhibitor	Inhibitor of	Company	Place	Product #	Experiment used in	conc.
Aprotinin	Unspecific serine proteases	Applichem	Unter-föhring, DE	A2132	Shear stress for bFGF release	200 U/ml
Abciximab	Integrin $\alpha_v\beta_3$	Lilly	Bad Homburg, DE	VL7140 001	Elastase treatment for bFGF release, FACS, Phospho-HSP27	0.5 µg/ml
SB202190	p38 MAPK	Upstate	Hamburg, DE	19-134	Elastase treatment for Phospho-HSP27	1 µM
Elastatinal	Elastase	SERVA	Heidelberg, DE	51798-45-9	Elastase treatment for FACS	2 µM
GF109203X	PKC inhibitor	Biosource	Solingen, DE	PHZ108 3	FACS	8 µM

Cell culture

Medium:

- DMEM (10%)

Buffers and solutions:

- PBS(+)
- PBS(-)
- Collagenase solution

Cell isolation

Porcine aortic endothelial cells (PAEC) were used throughout all the experiments. The isolation of the PAEC was done as described previously by Gloe et. al (1999). In short,

fresh aorta were obtained from the local slaughterhouse and kept in sterile in PBS(+) until the final preparation. Fat and connecting tissue was trimmed from the aorta before it was cut longitudinally thereby exposing the luminal surface. After washing in PBS(-), the vessels were placed into a frame with the luminal side facing up. Thereafter, they were incubated in a sterile collagenase solution for 20 min at 37 °C in a humidified incubator. Finally, culture medium was applied to wash off the endothelial cells, whereupon they were cultivated in standard plastic culture dishes in DMEM (10%) at 37°C and gassed with air enriched with 5 % CO_2.

Shear stress

Passages 2-3 of PAEC were used for application of shear stress either by the cone-and-plate technique or by the parallel plate system. The two systems are previously described by Gloe *et al.* (1999) and Koslow *et al.* (1986) and Frangos *et al.* (1987), respectively. The different systems were used for various reasons. The cone-and-plate apparatus needs less medium (7 ml) than the parallel plate system (50 ml), making the cone-and-plate system an advantage when inhibitors are administrated during the shear stress because less of the expensive inhibitors needs to be added. On the other hand, the cone-and-plate apparatus cannot be utilized for immuno-histochemistry experiments, since it is equipped with a glass plate of 10 cm in diameter which is too thick for the proper application of high magnification microscope lenses. Instead, the smaller glass plates of the parallel plate system (4.5 cm diameter) are much thinner and are therefore more suitable for microscopic analysis of cells grown on them.

Cone-and-plate shear apparatus

Media:

- DMEM (10%)
- DMEM (1%)

Buffer and solutions:

- Laminin type I

Inhibitor:

- Aprotinin

PAECs were seeded onto glass plates with a diameter of 10 cm pre-coated with laminin type I. The seeding of the cells onto a glass plate was done since plastic culture dishes were not suitable for shear stress experiment because they exhibited a high variability in the bottom thickness and were not perfectly planar.

After plating the endothelial cells onto the glass plate they were left in DMEM (10 %) until they were confluent (approximately 1-2 days). The confluent glass plates

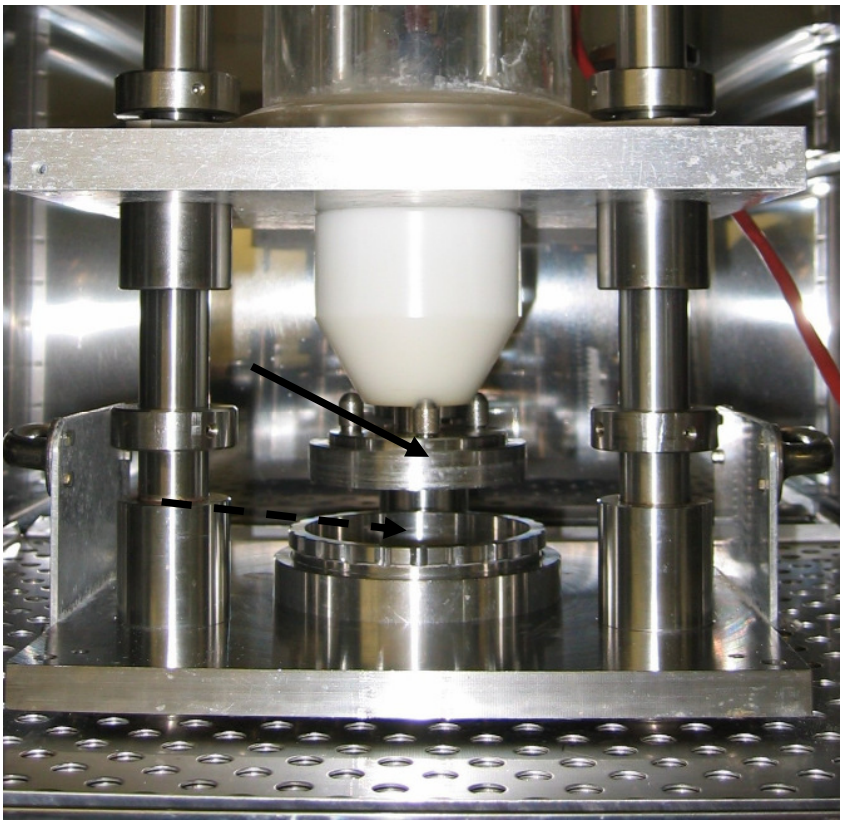


Figure 9: Cone-and-plate apparatus. Cells were plated onto a glass plate pre-coated with laminin I. When the cells were confluent, the glass plate was inserted into the lower chamber (dashed line). The upper part (the rotating cone (solid line)) was lowered close to the cells. By rotating, the medium (7 ml DMEM (1%) was set in motion creating a laminar shear stress on the cells.

were transferred into the cone-and-plate apparatus (see fig. 9), 7 ml DMEM (1 %) was added and a laminar shear stress of 16 dyn/cm^2 was applied to the endothelial cells for 2 hours at 37 °C.

In some experiments the unspecific serine protease inhibitor, aprotinin, was added to the medium during shear stress exposure. After shear stress, the supernatant was collected for measurement of bFGF release and elastase-like activity (see later description). The cells were lysed as described later.

Parallel-plate shear apparatus

Media:

- DMEM (10%)
- Leibovitz L-15 (1%)

Buffers and solutions:

- Laminin type I
- 3.7% formaldehyde

The parallel plate system was used in the case where the shear stress exposed cells should be analyzed by immuno-histochemistry. PAEC were seeded onto glass plates with a diameter of 4.5 cm pre-coated with laminin type I. The cells were allowed to adhere to the glass plate in DMEM (10 %). When the cells were confluent after approximately 1-2 days, the glass plate were placed in a gap of the lower plate of the parallel plate chamber and the upper plate of the camber was placed on top, the distance between both plates being determined by a Teflon gasket (fig. 10). The camber was placed on a heating block insuring a constant temperature of 37°C for the cells. Leibovitz L-15 medium (1 %) was allowed to flow over the cells creating a laminar flow. Leibovitz L-15 medium was used because the shear stress experiment was preformed outside a CO_2 incubator, i.e. in a carbon dioxide free environment. The Leibovitz L-15 medium is buffered by salts, free amino acids and galactose, which is contained in the medium and help to maintain physiological pH control.

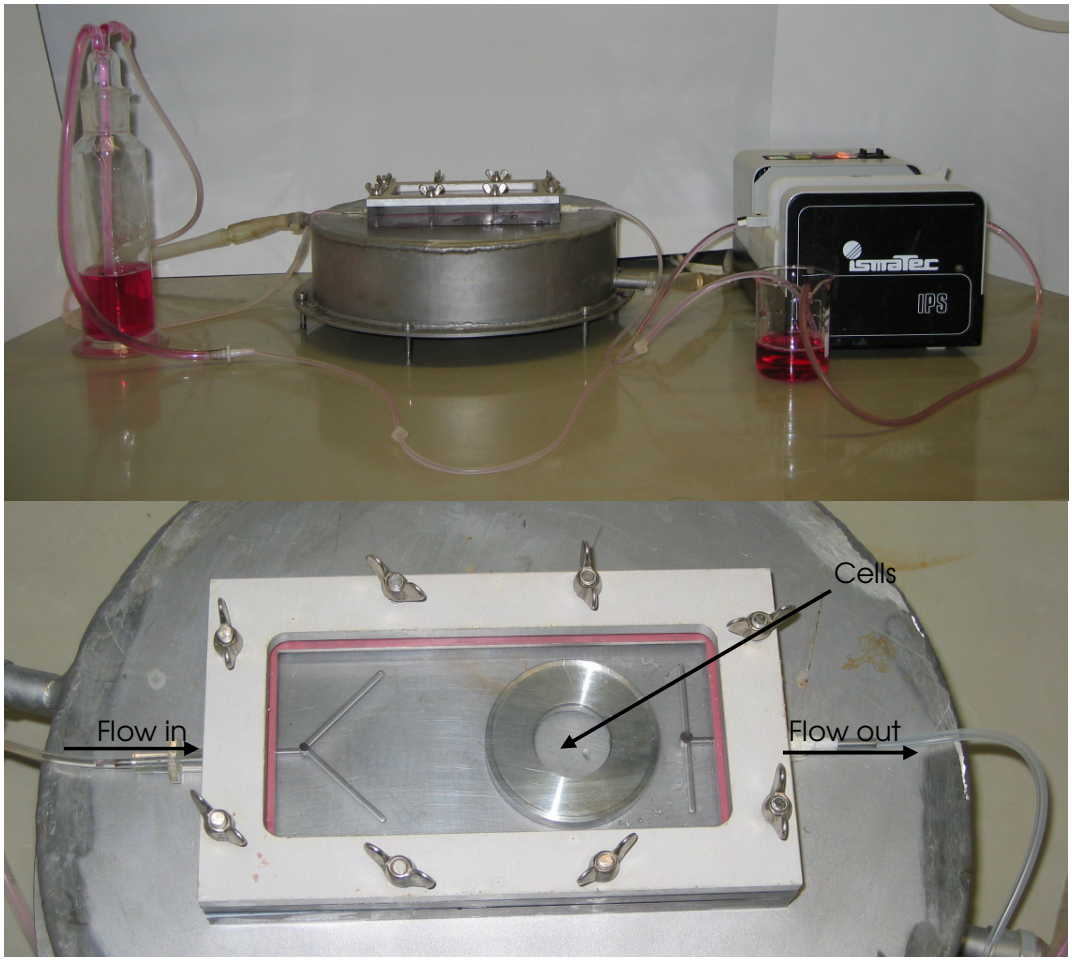


Figure 10: Parallel-plate system. A. The whole setup with the chamber containing the cells in the middle and the pump to the right which pumps the medium through the system. **B.** The chamber containing the cells between the upper and the lower plate. The medium is pumped from left to right passing the cells exposing them to a laminar shear stress.

In this shear stress system the shear rates over the glass plate with the cells is linear with flow rate. The velocity profile for laminar flow between parallel plates is given by the equation (Frangos *et al.*, 1987)

$$\tau = \frac{6Q\mu}{bh^2}$$

where Q is the flow rate (cm^3/s); μ is the viscosity (ca. 0.01 dyn s/cm^2); h is the channel height (12 mm); b is the slit width (56 mm); and τ is the shear stress (dyn/cm^2).

Since the chamber size is given, the only variably in our system is the flow rate (Q). At the maximum flow rate for our system the laminar flow was calculated to be 12 dyn/cm^2.

After 2 hours of shear stress exposure of the cells the glass plate containing the cells was quickly taken out of the chamber and fixed in 3.7% formaldehyde overnight at 4°C. The following day, the cells were stained for immuno-histochemistry (see section about immuno-histochemistry).

Elastase treatment

Medium:

- DMEM (1%)

Buffers and solutions:

- Elastase (0.5 U/ml)

Inhibitors:

- Elastatinal (2 µM)
- Abxicimab (0.5 µg/ml)
- SB202190 (1µM)

Passage 2-3 of PAEC was used for elastase treatment. The experiments were performed on static cells to which no shear stress was applied. For the acute experiment, the DMEM (10%) was exchanged for DMEM (1%).

To show that the exogenous elastase was functioning, control experiments were performed, where a specific substrate for elastase (MeOSuc-Ala-Ala-Pro-Val-pNA) in DMEM (1%) was subjected to increasing concentrations of elastase. In parallel, the substrate was incubated with elastase in combination with an elastase specific inhibitor, Elastatinal. Elastase, by cleaving the substrate, induced a change in the absorbance of the reaction solution, which was quantified in a spectrophotometer at a wave length of 450 nm.

In a first series of experiments, different concentrations of elastase were applied to the static endothelial cells (0; 0.1; 0.3; 0.5; 1.0 or 2.0 U/ml) for 2 hours at 37 °C. In all the following experiment an elastase concentration of 0.5 U/ml was used.

In further experiments, one of the following inhibitors or antibodies were added to the medium 1 hour prior to the beginning of the elastase treatment: Abciximab, which is an inhibitory antibody for GB IIbIIIa on plateletes and for the integrin $\alpha_v\beta_3$ on endothelial cells, an antibody against CD49f (integrin alpha 6) (1.0 µg/ml), an unspecific IgG (1.0 µg/ml) and the drug SB202190, which is an inhibitor of p38 MAP kinase.

ELISA for bFGF

Reagents:

- bFGF ELISA kit

The bFGF release was assessed by measuring the concentration of bFGF in the conditioned media from non-treated and treated cells. An ELISA kit was utilized and the protocol from the manufacturer was strictly followed. Briefly, 100 µl medium from control and treated cells was added to the ELISA wells together with a blocking agent and incubated for 2 hours at room temperature (RT). After washing 4 times with the washing buffer the wells were incubated with 200 µl bFGF conjugate for 2 hours at RT followed by a washing step. Subsequently, the substrate solution was added to the wells and after incubation for 30 min at RT, a stop solution was applied. The color reaction was measured using a spectrophotometer at 450 nm and at 620 nm. From the OD obtained the concentration in pg/ml could be calculated with the help of the standard curve.

Measurement of elastase activity

The specific substrate for elastase (MeOSuc-Ala-Ala-Pro-Val-pNA) was added to the conditioned medium from either static control cells or from shear stress exposed cells (16 dyn/cm^2, 2 hours). The cleavage of the substrate by elastase created a yellow

reaction solution which was measured at 450 nm. A standard curve was performed to convert the measured OD's into a concentration (mU/ml).

Western blots

Western blotting was used for determining translocation of HSP27 and phosphorylation of HSP27, p38 and p42/44 MAP kinases.

Reagents:

- BCA protein assay kit
- Enhanced chemoluminescence protein detection kit

Buffers and solutions:

- PBS(-)
- SDS-PAGE
- Lysis buffer
- Loading / sample buffer
- Running buffer
- Transfer buffer
- TBST (washing buffer)
- Na_3VO_4
- A- and B-blocking buffer

Antibodies:

- Anti-phospho-p38 MAP kinase antibody
- Anti-p38 MAP kinase antibody
- Anti-phospho-p42/44 MAP kinase antibody
- Anti-Actin
- HRP-conjugated anti-mouse antibody
- HRP-conjugated anti-rabbit antibody

Cell lysing

Cells exposed to shear stress or treated with elastase and their respective non-treated control cells, were washed with ice cold PBS(-) and lysed in lysis buffer. The cell were left on ice for 10 min to ensure proper cell lysing and the lysates were subsequently scraped together with a rubber policeman, passed through a 24G needle for three times and centrifuged for 10 min at 10,000 g at 4°C. The supernatants were used for further analyzing.

Protein measurement

Measurement of protein concentration was done by a BCA (bicinchoninic Acid) protein assay. The principle of this assay is similar to the Lowry procedure (Lowry *et al.*, 1951), in that a protein-cupper (Cu^{2+}) complex in alkaline conditions is formed. The Cu^{2+} is reduced to Cu^{1+} by BCA in this assay and folin in the case of the Lowry procedure. The amount of reduction is proportional to the protein content. BCA forms a purple-blue complex with Cu^{1+}, which was measured in a spectrophotometer at a wave length of 550 nm. The protein concentration was calculated from a standard curve generated with bovine serum albumin (BSA).

SDS-PAGE and protein transfer

Aliquots of the protein preparations (30 µg total proteins) were boiled for 5 min (100°C) and spinned donw before they were separated on a 10 or 12% SDS-PAGE. After separation of the proteins they were transferred to a nitrocellulose membrane, based on the design of (Towbin *et al.*, 1979), by semi-dry transfer technique. In this case a sandwich was made of 2-3 filterpapers soaked in transfer buffer, nitrocellulose membrane, gel and again 2-3 fitlerpapers. The proteins were blottet 1 hour at 0.8 mA / cm^2.

After the proteins were blottet onto the membrane, it was blocked with blocking buffer either with milk in cases where no phospho-specific antibodies were used or

BSA if phospho-specific antibodies were utilized, followed by incubation with the primary antibody (see table 2) in A- and B-blocking buffer, respetively, either for 2 hours at 37°C or overnight at 4°C. Unspecific bound antibody was washed off by TBST (3 x 10 min). The bound primary antibodies were detected by using HRP-conjugated anti-rabbit, anti-mouse or anti-goat IgG diluted in milk or BSA blocking buffer (see table 3) for 1 hour at RT. The bound secondary antibodies were viewed by using an enhanced chemoluminescence protein detection kit and x-ray films (Kodak). The detected bands were recorded and quantified with a videodensitometric system from Bio-Rad (München, Germany).

Immunoblot for phospho-p38 and phospho-p42/44

Activation of p38- and p42/44 MAP kinase was investigated by determining phosphorylation of p38 and p42/44 by Western blotting using phospho-specific antibodies. PAEC were either kept as non-treated control cells or treated with elastase. In some experiments the elastase treatment was done in addition with Abciximab, as described previously. 20 min prior to the elastase treatment, 100 µM Na_3VO_4 was added to the cells. Na_3VO_4 is an inhibitor of protein tyrosine phosphotases and was used to reduce the dephosphorylation of p38 and p42/44 during elastase treatment. After lysing the cells, the proteins were separated on a 10% SDS-PAGE. After blocking, the membranes with the transferred proteins were first probed with either a phospho-specific anti-p38 antibody or a phospho-specific anti-p42/44 antibody. To ensure equal loading, the membranes were reprobed with an anti-p38 antibody or an anti-actin antibody (see table 2), respectively. The mean phosphorylation of p38 (expressed as the ration between phospho-p38 and p38) or p42/44 (expressed as the ration between phospho-p42/44 and actin in control cells was set to 100% and all other treatment conditions were compared to this group.

HSP27 translocation assay

Buffers and solutions:

- PBS(-)
- 12% SDS-PAGE
- Lysis buffer (w/o Triton-X-100)
- Lysis buffer (w Triton-X-100)

Antibodies:

- Anti-HSP27 antibody
- Anti-PECAM antibody
- HRP-conjugated anti-rabbit
- HRP-conjugated anti-goat

PAEC seeded in 10 cm culture dishes were either kept as static non-treated control cells, treated with elastase or exposed to shear stress. After treatment, the cells were washed in ice cold PBS(-) and disrupted in 1 ml lysis buffer (w/o Triton-X-100). The lysates were ultra-centrifuged at 10^5 g at 4°C for 1 hour. The supernatants containing the cytosolic fraction were taken off and the pellets containing the particulate fraction were re-suspended in 300 µl lysis buffer (w Triton-X-100) and re-centrifuged at 10^4 g for 10 min at 4°C. The supernatants were collected and defined as the membrane fraction (Triton-X-100 soluble fraction) whereas the remaining pellet contained the Triton-X-100 insoluble fraction. The relative amounts of HSP27 in the membrane fraction were determined densitometrically by Western blotting using an anti-HSP27 antibody (see table 2). The membranes were re-probed with an anti-PECAM antibody to ensure equal loading. The mean amount of HSP27 (expressed as the ration between HSP27 and PECAM) in the membrane fraction in control cells was set to 100% and all other treatment conditions were compared to this group.

Self-made ELISA for measuring co-precipitation

Reagents:

- Washing buffer (bFGF ELISA kit)
- Stop solution (2N sulfuric acid; bFGF ELISA kit)
- TMB

Buffers and solutions:

- PBS(+)
- D-blocking buffer

Antibodies:

- Anti-HSP27 antibody
- Anti-phospho-HSP27 antibody
- HRP-conjugate bFGF antibody (bFGF ELISA kit)

For determining possible interaction between bFGF and HSP27, a self-made ELISA was established (figure 11). In this procedure, the wells of a 96 well plate were coated for 2 hours at RT with either a anti-HSP27 antibody or a phospho-specific HSP27 antibody both with a concentration of 20 µg/ml in PBS(+). After coating with either of the two antibodies, non-bound antibodies were washed off the wells with washing buffer and finally blocked with D-blocking buffer for 5 min at RT. Lysates from PAEC were added to the wells either natively or after denaturing (boiling for 5 min). The boiling was done to destroy the protein-protein interactions, which thereby

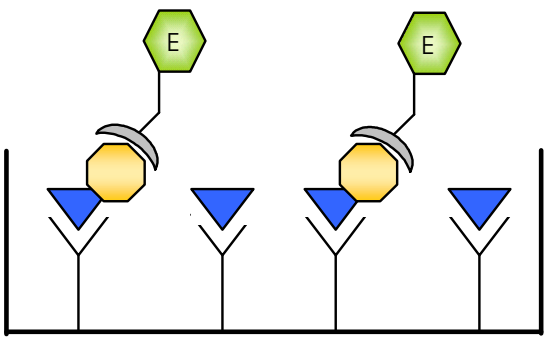


Figure 11: Co-precipitation of HSP27 and bFGF. The wells of a 96-well plate were coated with an anti-HSP27- or a phospho-specific HSP27 antibody. Lysates were added and the HSP27 or the phospho-HSP27 ( ) was captured by the antibody. If bFGF ( ) and HSP27 or phospho-HSP27 are interacting, bFGF will be pulled down together with the HSP27. The amounts of bFGF in the wells were detected by an anti-bFGF antibody directly HRP-coupled.

functioned as a negative control for HSP27 and bFGF interaction. Unbound proteins were washed off 3 times with washing buffer followed by incubation with an antibody against bFGF (50µl) directly HRP-conjugated to for 2 hours at RT. After three washings, a substrate for horseradish peroxidase, TMB was added to each well. After 30 min the reaction was stopped with 2N sulfuric acid forming a yellow reaction product, which was quantified by recording the optical density (OD) at 450 nm.

Phosphorylation assays for HSP27

Determination of HSP27 phosphorylation was associated with some difficulties. The most straightforward approach would have been the determination of the phosphorylation by Western blotting using a phospho-specific antibody. However, the phospho-specific antibodies used in this study were not suitable for Western blotting. Therefore, other methods had to be used for examining the HSP27 phosphorylation.

Phosphorylation determined by a self-made ELISA

Reagents:

- Washing buffer (bFGF ELISA kit)
- Stop solution (2N sulfuric acid; bFGF ELISA kit)
- TMB

Buffers and solutions:

- PBS(+)
- D-blocking buffer

Antibodies:

- Anti-HSP27 antibody
- Anti-phospho-HSP27 antibody
- HRP-conjugated anti-mouse antibody

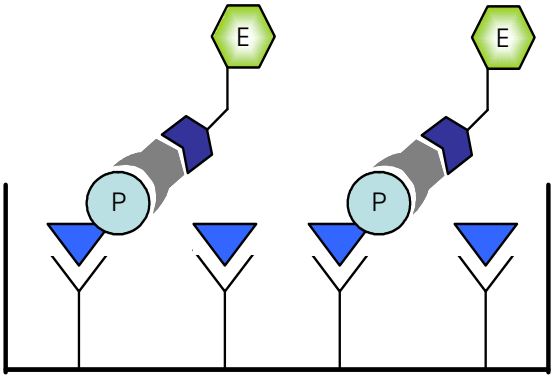


Figure 12: Detection of HSP27 phosphorylation. The wells of a 96-well plate were coated with a rabbit anti-HSP27 antibody. The total amount of HSP27 (blue triangles) in the lysate was thereby pulled down. A mouse phospho-specific HSP27 antibody (grey form) was detecting the phosphorylated HSP27 (light blue circles). A horseradish peroxidase conjugated secondary antibody (purple form) labeled the phopsho-specific HSP27 antibody.

The cell lysates used for this assay were from PAEC either kept as non-treated controls or treated with elastase or heat shock (42°C) for 2 hours each. In this self-made ELISA (figure 12), the wells of a 96 well plate were coated with a rabbit anti-HSP27 antibody (20 µg/ml in PBS(+)) (coating antibody) for 2 hours at RT.

As described previously, unbound coating antibodies were washed off by washing buffer (3 x 5 min) from R&D systems and the parts of the wells not bound by coating antibody were blocked with D-blocking buffer for 5 min at RT. Cell lysates were added to the coated wells and incubated for 2 hours at RT followed by a washing step to wash off unbound proteins. As detecting antibody a phospho-specific HSP27 antibody (20µg/ml in PBS(+)) was used, which was incubated for 2 hours at RT. An anti-mouse HRP-conjugated secondary detecting antibody was added to the wells and left for 1 hour at RT before washing 3x 5 min with washing buffer. The substrate for horseradish peroxidase, TMB, was added to the wells for 30 min at RT in dark and the reaction was terminated by 2N sulfuric acid creating a yellow the reaction solution which could be read at 450 nm in a spectrophotometer and thereby the changes in phosphorylation status of HSP27 was expressed as changes in OD.

Phosphorylation determined by immuno-precipitation

Reagents:

- Protein-A magnetic coupled micro-beads

Buffers and solutions:

- 12% SDS-PAGE
- Lysis buffer (w Triton-X-100)
- SDS sample/loading buffer

Antibodies:

- Anti-phospho-serine antibody
- Anti-HSP27 antibody
- HRP-conjugated anti-rabbit antibody

Cell lysates were prepared, as described previously, from PAEC treated with or without elastase. Protein aliquots of 300 µg each were mixed with a phospho-specific serine antibody (0.25 µg/100 µg protein) and protein-A magnetic coupled micro-beads (50µl) for 30 min on ice. The mixtures were loaded onto MACS columns which were placed onto a strong magnet (figure 13). The mixtures were slowly passing through the columns, and the protein-antibody-magnetic-bead complexes were retained in the columns. When all of the solution had passed through the columns they were washed 3 times with lysis buffer (1 ml). 20 µl of hot (95°C) SDS sample/loading buffer (see solutions in the Western blot section) was added to the column and incubated for 5 min. Subsequently, 50 µl hot sample/loading buffer was

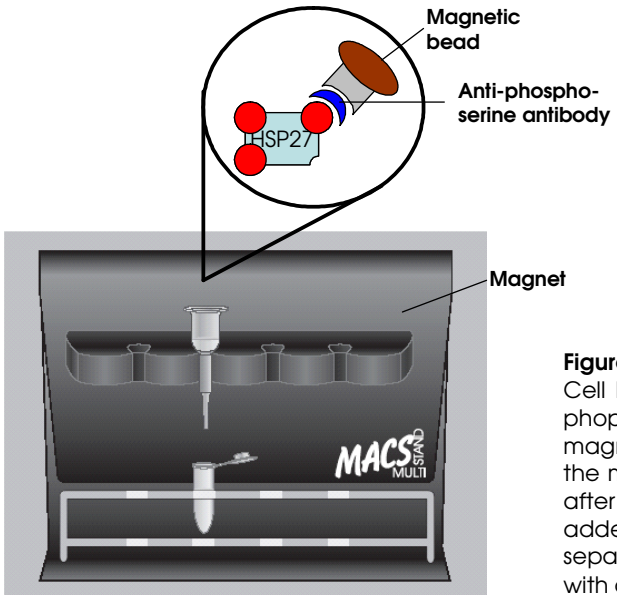


Figure 13: Immuno-precipitation by MACS columns. Cell lysates were incubated together with an anti-phopsho-serine antibody and protein-A coupled magnetic beads for 30 min on ice. Subsequently, the mixtures were passed through the columns and after washing, hot SDS sample/loading buffer was added to the columns. The eluted proteins were separated on a 12% SDS-PAGE and immuno-blotted with an anti-HSP27 antibody.

applied to the columns and the retained proteins was thereby eluted from the columns. Eluates were separated by 12% SDS-PAGE and immuno-detection was done with an anti-HSP27 antibody. The mean phosphorylation of HSP27 of control cells was set to 100% and all other treatment conditions were compared to this group.

Phosphorylation determined by isoelectric focusing

Buffers and solutions:

- IEF-gel
- IEF solubilization buffer
- Overlay buffer
- Cathode buffer
- Anode buffer
- IEF washing buffer
- Transfer buffer
- A-blocking buffer

Antibodies:

- Anti-HSP27 antibody
- HRP-conjugated anti-mouse

Isoelectric focusing (IEF) is electrophoresis in a pH gradient set up between cathode and anode. Proteins will be positively charged at pH values below their isoelectric point (IpH) and negatively charged above. This means that proteins will migrate towards their IpH. Phosphorylation of a protein increases its negative charge and thus increases the mobility of the protein towards the acidic (anode) region of the IEF gel.

The following protocol is based upon (Ploegh, 1995). Porcine aortic endothelial cells exposed to shear stress for 0, 30, 60 or 120 min. were after treatment lysed in lysis buffer containing the non-ionic detergent Triton-X-100, as described previously. To obtain reproducible separations of proteins by charge, the ionic strength of the

samples must be as low as possible and hence the salt concentration was reduced by dialyzing each sample in dialyzing tubes (10 kDa cut-off) by centrifugation for approximately 30 min at 4°C. The sample volume was thereby reduced by 85%. The protein samples were subsequently diluted 1:4 with IEF solubilization buffer and loaded onto a 13.5% IEF-gel containing urea and ampholytes. The gel was casted in the large electrophoresis chamber from Bio-Rad (16 cm x 18 cm x 0.1 cm), also used in Western blotting. The stacking gel normally used in protein electrophoresis was omitted. After loading of the samples, an overlay buffer was put on top of the samples to avoid contact between proteins and the cathode buffer, which was filled in the upper chamber. The lower chamber was filled with the anode buffer. A voltage of 400 Volts was applied to the gel over night and the following day the voltage was increased to 600 Volts for 1 hour.

For blotting the proteins onto a nitrocellulose membrane it was necessary to remove the non-ionic detergent from the gel, since it reduces the amount of proteins transferred to the membrane. Furthermore, SDS has to be bound to the protein in the gel in order to transfer them from the gel to the membrane. Both tasks were accomplished by washing the gel in washing buffer (5 x 10 min). After washing, the proteins were transferred to a nitrocellulose membrane by semi-dry transfer technique, as described above. The membrane was blocked with A-blocking buffer followed by incubation with an anti-HSP27 antibody in A-blocking buffer. The bound primary antibodies were detected by a HRP-conjugated anti-mouse antibody and viewed as described for Western blot. The data are expressed as the procentage of the amount of phosphorylation out of the total amount of detected protein within each treatment.

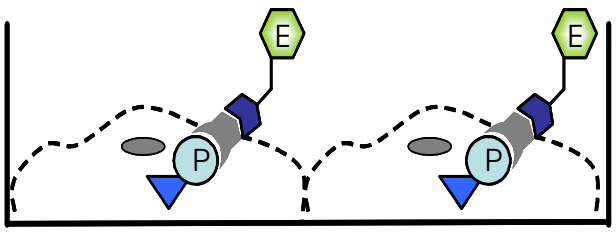


Figure 14: PACE for measurement of HSP27 phosphorylation. The principles in this assay are the same as described previously (see the section "phosphorylation determined by a modified ELISA").The new with this method is however, that the detection of the phospho-HSP27 is done within the cells instead of utilizing cell lysate.

Phosphorylation determined by Phosphospecific antibody Cell-Based ELISA (PACE)

Media:

- DMEM (10%)
- DMEM (1%)

Reagents:

- TMB

Buffers and solutions:

- PBS(-)
- PBS/Triton
- 4% formaldehyde
- H_2O_2
- C-blocking buffer
- E-blocking buffer

Antibodies:

- Anti-phospho-HSP27
- HRP-conjugated anti-rabbit

The following protocol is based upon Versteeg *et. al* 2000 (Versteeg *et al.*, 2000) (figure 14). PAEC were seeded in a 96-well plate. When they were confluent, the medium was changed from DMEM (10%) to DMEM (1%). The cells were kept as non-treated controls or treated with elastase for 2 hours at 37°C, as described previously. After treatment, the cells were fixed with 4% formaldehyde for 20 min at RT and washed (3 x 5 min) with PBS/Triton. H_2O_2 (0.6%) in PBS/Triton was added for 20 min to quench the endogenous peroxidase. The cells were washed two times in PBS/Triton, blocked with E-blocking buffer for 1 hour and subsequently incubated overnight at 4°C with an anti-phospho-HSP27 antibody diluted in C-blocking buffer. The next day,

the cells were washed (3 x 5 min) with PBS/Triton and incubated with a HRP-conjugated anti-rabbit antibody in C-blocking buffer for 1 hour at RT. The cells were washed (3 x 5 min) with PBS/Triton and twice with PBS(-). Subsequently, the cells were incubated with TMB in dark, creating a bluish solution which was measured in a spectrophotometer at a wave length of 720 nm after 30 min. The mean OD in control cells was set to 100% and all other treatment conditions were compared to this group.

Fluorescence-Activated Cell Sorter (FACS)

Reagents:

- CellWASH
- Propidium iodide
- Silicone solution®

Buffers and solutions:

- Elastase
- Citrate buffer
- 10% formaldehyde

Antibodies:

- Anti-bFGF antibody
- FITC-labled anti-rabbit antibody

Inhibitiors:

- Abxicimab
- Elastatinal
- GF109203X

FACS was used for analyzing the amount of bFGF on the cell surface. Basic FGF is a cytosolic protein, however, it is in the literature suggested that bFGF is released

through the plasma membrane (Engling *et al.*, 2002;Backhaus *et al.*, 2004;Nickel, 2003). If this would be correct, it should be hypothesized that an anti-bFGF antibody would be able to recognize the bFGF protein sticking out of the membrane after elastase treatment (figure 15). By labeling the first antibody with a fluorescence labled secondary antibody the number of cells exposing bFGF on their surface could be measured by flow cytometry. To investigate whether the elastase acts via the matrix or directly on the cell or its integrins, two different protocols were followed, one for cells attached to the matrix during treatment and one for cells kept in suspension during treatment.

Protocol for adherent cells

Passage 2-3 of PAEC was seeded in a 24 well plate until they were app. 90% confluent. The cells were treated with elastase (as described previously). 30 min prior to the end of the treatment, propidium iodide (PI, 1:100) was added to the cells. PI is a compound, which is binding to double-stranded DNA, meaning it has to cross the plasma membrane, which it only can do in non-viable cells. Hence, the PI staining was done in order to make sure that only non-permeabilized cells were used for the measurement of the bFGF cell surface staining. As positive control for permeabilized cells the PKC inhibitor, GF109203X, was used, since it in high concentrations causes cell damage.

After 2 hours of elastase exposure, the medium was taken off and the cells were washed with 200 µl citrate buffer followed by incubation in the same amount of citrate buffer at 37°C until the cells were detached from the plate (approximately 1 hour). Citrate buffer was used as a non-enzymatic form of detachment of cells, which in this situation was preferable, since an enzymatic reaction might influence

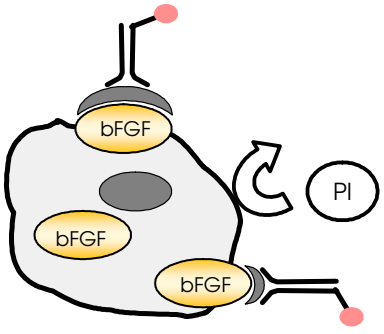


Figure 15: FACS analysis of bFGF cell surface localization. Anti-bFGF antibodies were detecting the bFGF only on the membrane of non-permeabilized cells (propidium iodide (PI) negative). The bFGF antibodies were labeled by a fluorescent conjugated secondary antibody, which could be measured by flow cytometry.

the bFGF distribution in the cells. After detachment, the cells in the citrate buffer were fixed in 100 µl 10% formaldehyde for 10 min at RT. For washing off the formaldehyde, 1 ml cellWASH was added to each tube and centrifuged for 5 min at 3000 rpm at RT. The Eppendorf tubes were turned upside-down to pour out the liquid leaving the cells in the tubes in app. 100 µl liquid. An anti-bFGF antibody in PBS was added to the cells and incubated for 30 min at RT. To wash off unbound antibodies, 1 ml CellWASH was added to the each sample, centrifuged for 5 min. at 3000 rpm and again the Eppendorf tubes were turned to pure out the liquid leaving the cells in app. 100 µl liquid. As secondary antibody, a FITC-labled anti-rabbit antibody in PBS(-) was used to detect the anti-bFGF antibodies, which were adhering to the bFGF on the cell surface (figure 14). The cells were incubated with the secondary antibody for 30 min in dark at RT and subsequently, the cells were washed in 1 ml CellWASH, centrifuged and the liquid discharged. Finally, 300 µl CellWASH was added to each sample before measuring in the flow cytometer. The argon laser in the flow cytometer excited the FITC with a wave length of 488 nm and the emission wave length of 510 nm was measured. In some experiments, the elastase treatment was done in combination with the inhibitors Abciximab or Elastatinal added to the cells 1 hour prior to elastase treatment.

The mean fluorescence in control cells was set to 100% and all other treatment conditions were compared to this group.

Protocol for suspended cells

Cells were seeded as described for adherent cells. Before elastase treatment, the cells were detached from their matrix with 200 µl citrate buffer (app. 1 hour at 37°C). Eppendorf tubes were coated with silicone, by submersing the tubes in silicone solution® containing isopropanol for 10 min at RT, followed by washing 3 times in distilled water. The coating was done in order to prevent cells from adhering to the Eppendorf tube during the elastase treatment and thereby ensuring that the cells were kept in suspension. The cells were centrifuged down (5 min at 3000 rpm at RT) and the citrate buffer was carefully discharged. DMEM medium (200 µl) containing 1% FCS was added to each Eppendorf tube containing the cells. Elastase (0.5 U/ml) was added to each tube and left for incubation at 37°C for 2 hours.

The following steps were the same as described for adherent cells after elastase treatment.

Fast Activated Cell-Based ELISA (FACE)

Media:

- DMEM (10%)
- DMEM (1%)

Reagents:

- TMB

Buffers and solutions:

- PBS(-)
- 4% formaldehyde
- C-blocking buffer
- F-blocking buffer

Antibodies:

- Anti-bFGF
- HRP-conjugated anti-rabbit

With some modifications of the PACE method, it was possible to determine the amount of bFGF on the cell surface. This assay was called Fast Activated Cell-Based ELISA (FACE; adapted form the company Active Motif; Rixensart, Belgium) instead of PACE, since this assay has nothing to do with phosphorylation. The protocol is basically the same as described for PACE, however, the cells should not be permeabilized and hence, no Triton-X-100 was used. Briefly, PAEC were seeded in a 96-well plate, stimulated with elastase for 2 hours or kept as controls and fixed with 4% formaldehyde. The cells were washed 3 times in PBS(-), blocked with F-blocking buffer and subsequently incubated with an anti-bFGF antibody in C-blocking buffer overnight at 4°C. The next day, the cells were washed with PBS(-) and incubated with

a HRP-conjugated anti-rabbit antibody in C-blocking buffer for 1 hour at RT. After three times wash, the cells were incubated with TMB in dark and the reaction was measured after 15 min at a wave length of 720 nm. The mean OD in control cells was set to 100% and other treatments were compared to this group.

Immuno-histochemistry

Media:

- DMEM (10%)
- DMEM (1%)
- Leibovitz L-15 medium (1%)

Buffers and solutions:

- Elastase
- Laminin type I
- 3.7% formaldehyde
- D-blocking buffer

Antibodies:

- Anti-HSP27 antibody
- Anti-bFGF antibody
- FITC-labeled anti-rabbit antibody

PAEC, passage 2-3, was used for immuno-staining, which was done on either non-treated (control), shear stress- or elastase exposed cells. In case of shear stress experiments, the cells were seeded onto glass plates with a diameter of 4.5 cm pre-coated with laminin type I. The shear stress experiments were performed in the parallel-plate system, as described previously. The control cells were kept static for as long as the shear stress treatment. In the case of elastase exposure, the cells were seeded on 1 cm cover slips pre-coated with laminin type I in a 24 well plate. The elastase treatment (0.5 U/ml, 2 hours) was performed as described previously.

After treatment with either shear stress or elastase, the medium was discarded and the cells were fixed in 3.7% formaldehyde overnight at 4°C. The next day, the formaldehyde was washed off and D-blocking buffer was used to prevent unspecific binding of antibody to the cover slips. After 20 min of blocking, the first antibody (either an anti-bFGF- or an anti-HSP27 antibody) was applied to the cells diluted in D-blocking buffer for 1 hour at RT. Unbound and unspecific bound first antibody was washed of in PBS(-) before a FITC-labeled anti-rabbit diluted in D-blocking buffer was applied to the cells and incubated in dark for 30 min at RT. Finally, the cells were washed and the cover slips with the cells were inverted and glued to an object glass. Subsequently, the cells were visualized in a Zeiss LMS 410 invert laser scan microscope (Ziess, Munich, Germany) with an excitation wavelength of 543 nm and an emission wavelength of 578 nm. As controls, cells stained with only the secondary antibody were used.

Statistic

All the statistical analysis was performed with Sigma Stat version 2.0. Data are expressed as mean ± SEM. Differences between values were considered statistically significant if $p \leq 0.05$. In cases where only two values are compared, a paired t-test was utilized for statistical testing. When more than two experiments were compared, a one-way ANOVA on Ranks was performed to test for differences compared to control cells. To identify the differences a Dunn's method was used.

Results

The role of proteases in bFGF release

Shear stress, elastase activity and bFGF release

A 3 fold higher elastase activity was observed in the conditioned medium from shear stress exposed porcine aortic endothelial cells (laminar shear stress (16 dyn/cm^2)) compared to static control ($p<0.05$; n=6; figure 16). Likewise, the bFGF concentration in the conditioned medium from shear stress exposed cells was about 5-fold higher than in the supernatant of static control cells ($p<0.05$; n=9). This increase was abolished upon unspecific inhibition of serine proteases by aprotinin ($p<0.05$; n=9; figure 17).

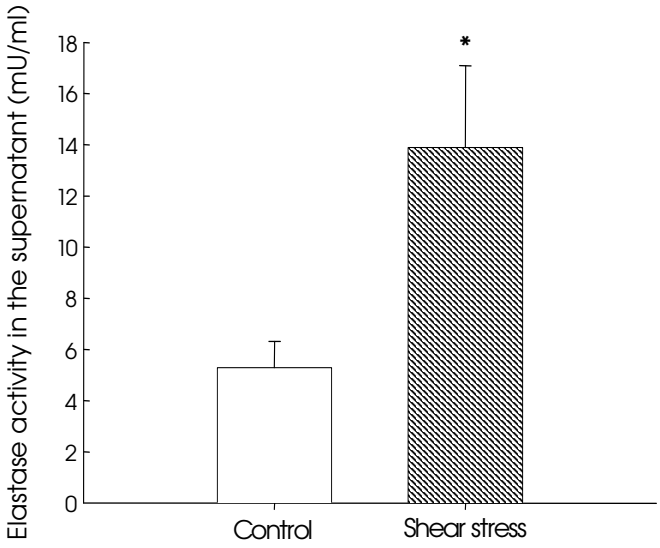


Figure 16. Effect of shear stress on elastase activity. Exposure to shear stress significantly increased the elastase activity in the supernatant of PAEC. A specific substrate for elastase (MeOSuc-Ala-Ala-Pro-Val-pNA) was applied to the conditioned medium of either static control - or shear stress exposed porcine aortic endothelial cells. By cleavage of the substrate the solution turned yellow allowing to measuring the reaction product at 450 nm. A standard curve was performed to convert the measured optical densities (OD) into a concentration (mU/ml). The bar graphs represent means ± SEM. *p<0.05 compared to control. n=6.

Elastase treatment and bFGF release

It has previously been shown that elastase is a protease able to induce a release of bFGF when applied to endothelial cells (Buczek-Thomas & Nugent, 1999). Therefore, in the present study experiments were performed with application of porcine pancreatic elastase to PAEC. As control, it was shown that conventional elastase as obtained from SERVA (see methods) was able to cleave the same elastase specific substrate (MeOSuc-Ala-Ala-Pro-Val-pNa) as the endogenous elastase detected in

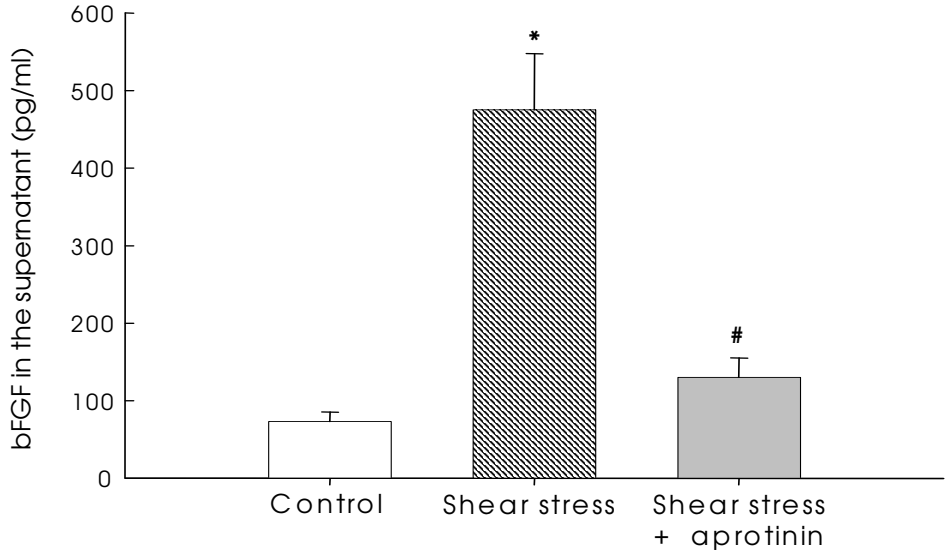


Figure 17. Effect of shear stress on bFGF release. The shear stress induced bFGF release was abolished by protease inhibition. Porcine aortic endothelial cells were either left static (control) or exposed to shear stress (16 dyn/cm^2) for 2 hours. Basic FGF concentration was measured in the conditioned medium. The last bar represents the bFGF release after shear stress exposure in combination with an unspecific serine protease inhibitor, aprotinin. The bar graphs represent means ± SEM. *p<0.05 compared to control. #p<0.05 compared to shear stress. n=9.

the supernatant of shear stress exposed cells. The cleavage occurered in a concentration dependent manner and was inhibited by the specific elastase inhibitor, Elastatinal (figure 18). Therefore, this conventional elastase was used to assess the possibility that the increased elastase activity after shear stress has a functional role regarding bFGF release. Static endothelial cells were incubated with different concentrations of elastase (0; 0.1; 0.3; 0.5; 1.0 and 2.0 U/ml), resulting in a

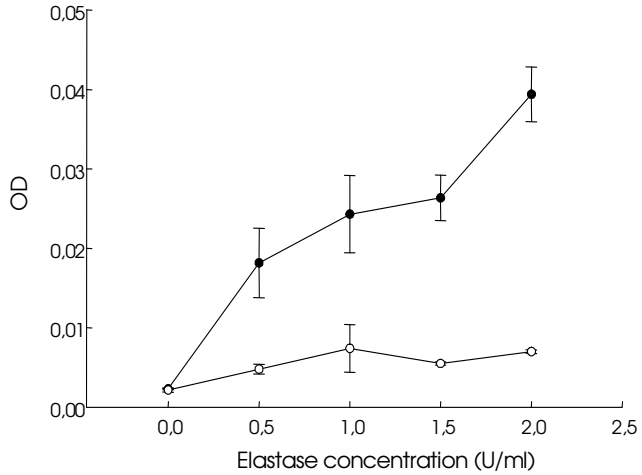


Figure 18. Activity of conventional elastase. Conventional porcine pancreatic elastase cleaved the same substrate (MeOSuc-Ala-Ala-Pro-Val-pNA) as utilized in the endogenous elastase activity assay in a concentration dependent manner (n=4). In some experiments (n=2) a specific inhibitor of elastase, elastatinal, was added to the reaction solution. Black circles represent elastase treatment; white circles represent elastase treatment in combination with elastatinal.

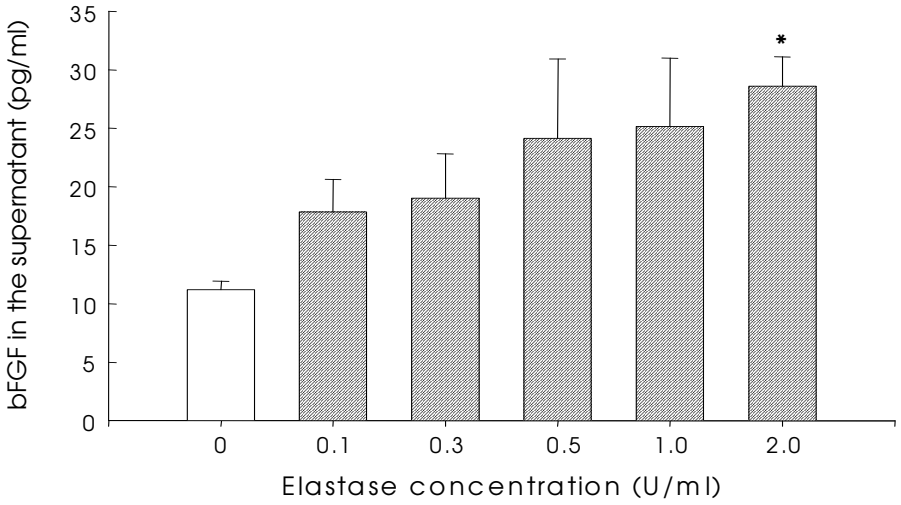


Figure 19. Basic FGF release after elastase treatment. Exogenously added elastase increased the bFGF release from static cells in a concentration dependent manner. Porcine pancreatic elastase was added to the medium of static endothelial cells at increasing concentrations (0; 0.1; 0.3; 0.5; 1 and 2 U/ml). After 2 hours, the bFGF concentration (pg/ml) in the conditioned medium was measured (ELISA). Bar graphs represent means ± SEM. *p<0.05 compared to non-treated control cells (0 U/ml). n=9.

concentration depended release of bFGF from the cells ($p<0.05$, 2 U/ml compared to 0 U/ml; n=9; figure19A). Note that an elastase concentration of 0.5 U/ml resulted in a 2 fold higher bFGF release compared to non-treated control cells ($p<0.05$; n=8; figure 20A). At this elastase concentration, the cells looked healthy and did not stain positive for propidium iodide (PI) (no significant difference between untreated (control) and treated cells (elastase); $p<0.01$ compared to permeabilized cells (perm); n=3-5; figure 20B). Since it is favorable to use as low a concentration of the enzyme as possible, 0.5 U/ml elastase was utilized in all the following experiments unless stated otherwise.

The above results indicate that elastase induces bFGF release in a way other than membrane damage. To investigate the mechanism which could be involved in the release, cells were treated with elastase in combination with an inhibitory antibody against integrin $\alpha_v\beta_3$ (Abxicimab), based on a previous study, showing that shear stress induced bFGF release could be abolished by inhibition of the integrin $\alpha_v\beta_3$ (Gloe *et al.*, 2002). This integrin inhibition showed a reduction in the elastase mediated bFGF release ($p<0.05$, elastase (Ela) + Abxicimab (Abx) compared to elastase; n=7; figure 21). In contrast, an inhibitory antibody against integrin α_6 (CD49) or an unspecific IgG did not cause any reduction in the bFGF release (NS; n=4).

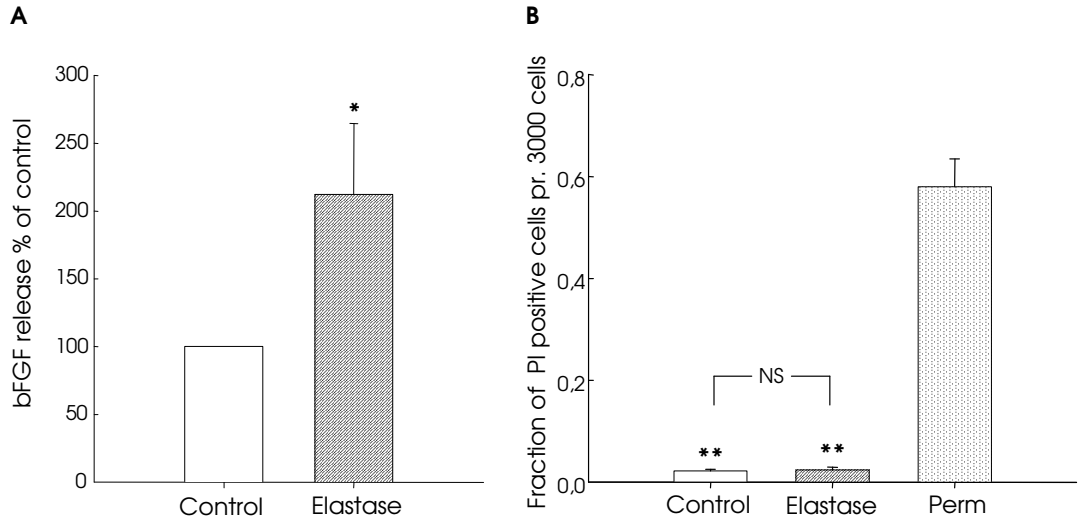


Figure 20. Elastase treatment with a concentration of 0.5 U/ml. *A.* Exogenously added elastase significantly increased the bFGF release from static cells. Cells were either left untreated (control) or treated with porcine pancreatic elastase (0.5 U/ml). The concentration of bFGF (pg/ml) in the conditioned medium was measured after 2 hours of treatment. Bar graphs represent the bFGF release expressed as % of the level before elastase treatment (control). *p<0.05 compared to control. n=8. ***B***. Exogenously added elastase caused no cell damage, since treated cells were not PI positive. Cells were either kept as non-treated control cells, treated with porcine pancreatic elastase (0.5 U/ml, 2 hours) or with GF109203X (permeabilized (perm)), a PKC inhibitor, which in high concentrations cause cell damage (functions as a positive control for PI staining). The bar graphs represent the fraction of cells which were stained positive for PI pr. 3000 counted cells. **p<0.01 compared to permeabilized cells (perm). NS: not significant difference between control and elastase treated cells. n=3-5.

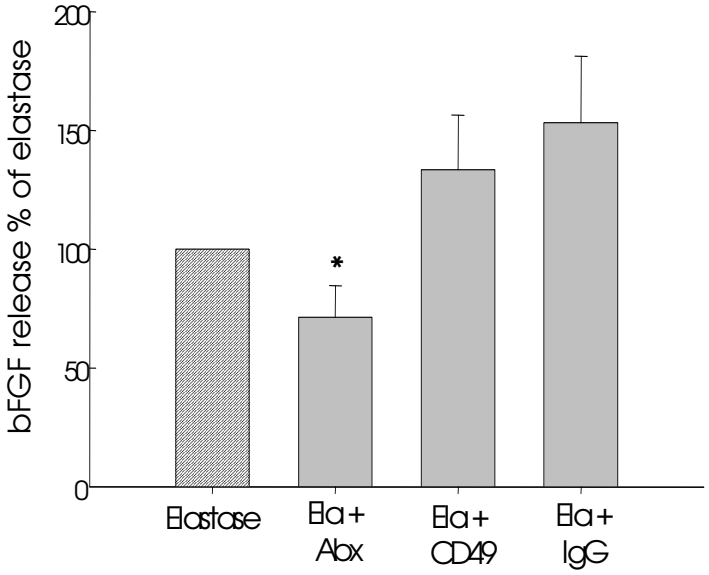


Figure 21. Selective inhibition of elastase mediated bFGF release by an $\alpha_v\beta_3$ antibody. Porcine aortic endothelial cells were either treated with porcine pancreatic elastase (0.5 U/ml, 2 hours) alone or in combination with the inhibitory antibodies against integrin $\alpha_v\beta_3$ (Abxicimab (Abx) 0.5 µg/ml), integrin α_6 (CD49, 1.0 µg/ml) or an unspecific IgG (1.0 µg/ml). The bFGF concentration (pg/ml) was subsequently measured in the conditioned media. Ela denotes elastase. The bar graphs represent the bFGF release expressed as % of elastase treatment. *p<0.05 compared to elastase. n=4-7.

The bFGF translocation in endothelial cells

bFGF translocation towards the cell membrane

To evaluate the possibility that bFGF is released through the membrane, immuno-staining of bFGF in control, shear stress exposed or elastase treated cells was performed. Non-treated control cells displayed primarily a diffuse perinuclear distribution of bFGF, whereas shear stress exposed as wells as elastase treated cells in addition clearly displayed a marked staining of the cell membrane. Figure 22 show representative pictures as they appeared in 3 independent experiments.

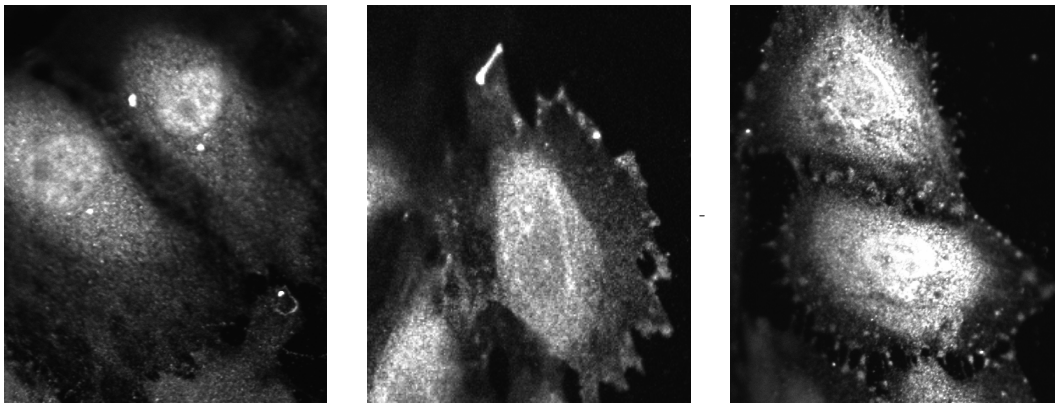


Figure 22. Immuno-histochemisty of bFGF. Subcellular localization of bFGF in PAEC visualized by immuno-histochemistry showing translocation of bFGF towards the plasma membrane upon shear stress and elastase treatment. Direct immuno-fluorescence was performed on cells kept as non-treated control cells or cells exposed to shear stress (16 dyn/cm^2, 2 hours) or treated with elastase (0.5 U/ml, 2 hours). After treatment, cells were fixed, permeabilized and stained with a polyclonal rabbit anti-bFGF antibody followed by incubation with a fluorescent-labeled anti-rabbit antibody. Representative pictures from 3 independent experiments.

Elastase-mediated bFGF translocation

By utilizing the FACE method, the cell surface localization of bFGF after elastase treatment was confirmed. Treatment of cells with increasing concentration of elastase (n=5) showed a concentration-dependent increase in the amount of bFGF

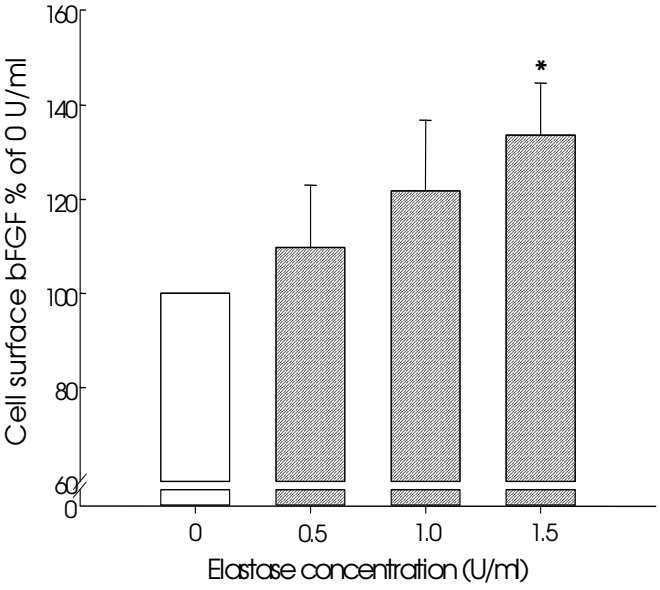


Figure 23. Quantitative analysis of bFGF cell surface localization by Fast Activated Cell-based ELISA (FACE). Exogenously added elastase increased the amount of bFGF at the cell surface in a concentration dependent manner. Porcine aortic endothelial cells were seeded in a 96-well plate and subjected to different concentrations of porcine pancreatic elastase (0, 0.5, 1 and 1.5 U/ml). After 2 hours of treatment, the cells were fixed and kept non-permeabilized, whereby the bFGF on the cell membrane could be detected by a specific anti-bFGF antibody. The bar graphs represent the bFGF on the cell surface expressed as % of non-treated control. *p<0.05 compared to non-treated cells (0 U/ml). n=5.

at the cell surface, with a significantly higher cell surface staining at an elastase concentration of 1.5 U/ml ($p<0.05$, 1.5 U/ml compared to 0 U/ml; figure 23).

Matrix-dependent bFGF translocation

In addition to FACE, FACS analysis further verified, that there was significantly more bFGF at the cell surface after elastase treatment compared to non-treated control cells (1.5 fold; $p<0.05$; n=13; figure 24A). The amount of cell surface bFGF was reduced by a specific elastase inhibitor, elastatinal, which was added to the medium during elastase treatment ($p<0.05$ elastase (Ela) + elastatinal compared to elastase treatment; n=9; figure 24A). Furthermore, the elastase mediated translocation was significantly reduced by inhibition of integrin $\alpha_v\beta_3$ ($p<0.05$ elastase (Ela) + Abxicimab (Abx) compared to elastase treatment; n=13; figure 24A). In contrast, no translocation of bFGF was observed when the endothelial cells were detached from the matrix and kept in suspension during elastase treatment (NS; n=7; figure 24B).

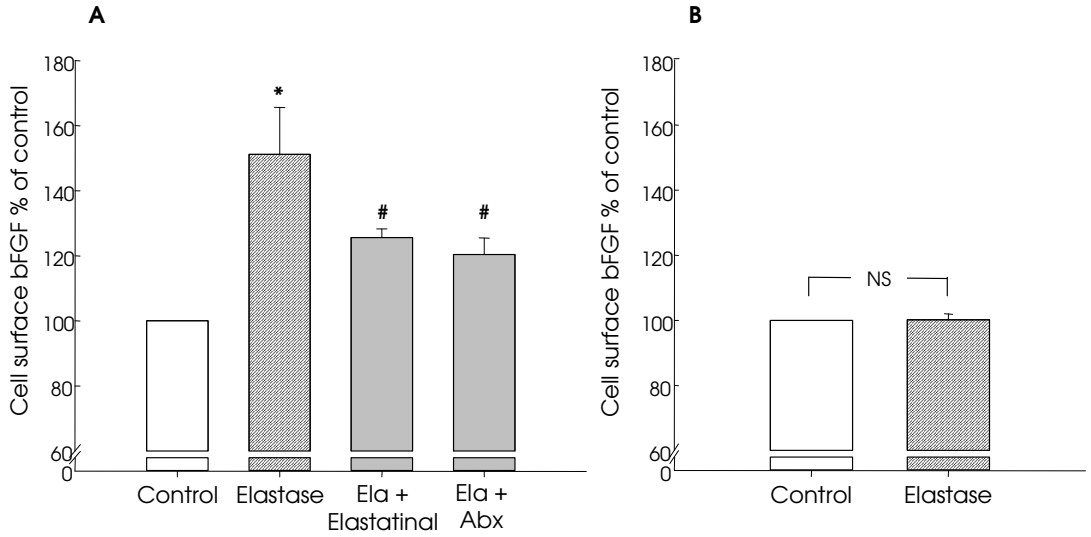


Figure 24. Flow cytometry analysis of bFGF cell surface localization. ***A.*** Inhibition of elastase or integrin $\alpha_v\beta_3$ reduced the elastase induced cell surface localization of bFGF. The bFGF cell surface expression was detected by the corresponding FITC-labeled antibody in untreated (control), elastase treated (0.5 U/ml, 2 hours), elastase + elastatinal (specific inhibitor of elastase, 2µM) or elastase + Abxicimab (Abx) (inhibitor of integrin $\alpha_v\beta_3$, 0.5 U/ml) treated cells. The inhibitors were added to the cells 1 hour prior to the elastase treatment. After treatment the cells were detached from the culture plates without using a protease-based protocol and kept non-permeabilized. Ela denotes elastase. The bar graphs represent bFGF cell surface staining expressed as % of non-treated control level. *$p<0.05$ compared to control, n=13; #$p<0.05$ compared to elastase. n= 9-13. ***B.*** Treatment with exogenous elastase did not cause bFGF translocation to the plasma membrane in cells kept in suspension. PAEC was detached with citrate buffer before elastase treatment and kept in suspension during treatment. The bFGF on the cell surface was measured in the suspended cells. The bar graphs represent bFGF cell surface staining expressed as % of non-treated control level. NS denotes not significant. n=7.

Involvement of HSP27 in bFGF release

HSP27 phosphorylation

The phosphorylation of HSP27 during shear stress exposure of endothelial cells has previously been shown (Azuma *et al.*, 2001;Li *et al.*, 1996) and was here verified by isoelectric focusing (IEF). Figure 24A shows four different bands separated on a pH-gradient gel. The upper band represents the non-phosphorylated isoform (NP), whereas the three lower bands represent the mono-, the di- and the tri-phosphorylated isoforms denoted P_1, P_2, and P_3, respectively. The individual bands were quantified by densitometry and the percentage of total phosphorylation was calculated and graphically illustrated in figure 24B (n=2). Elastase treatment was also

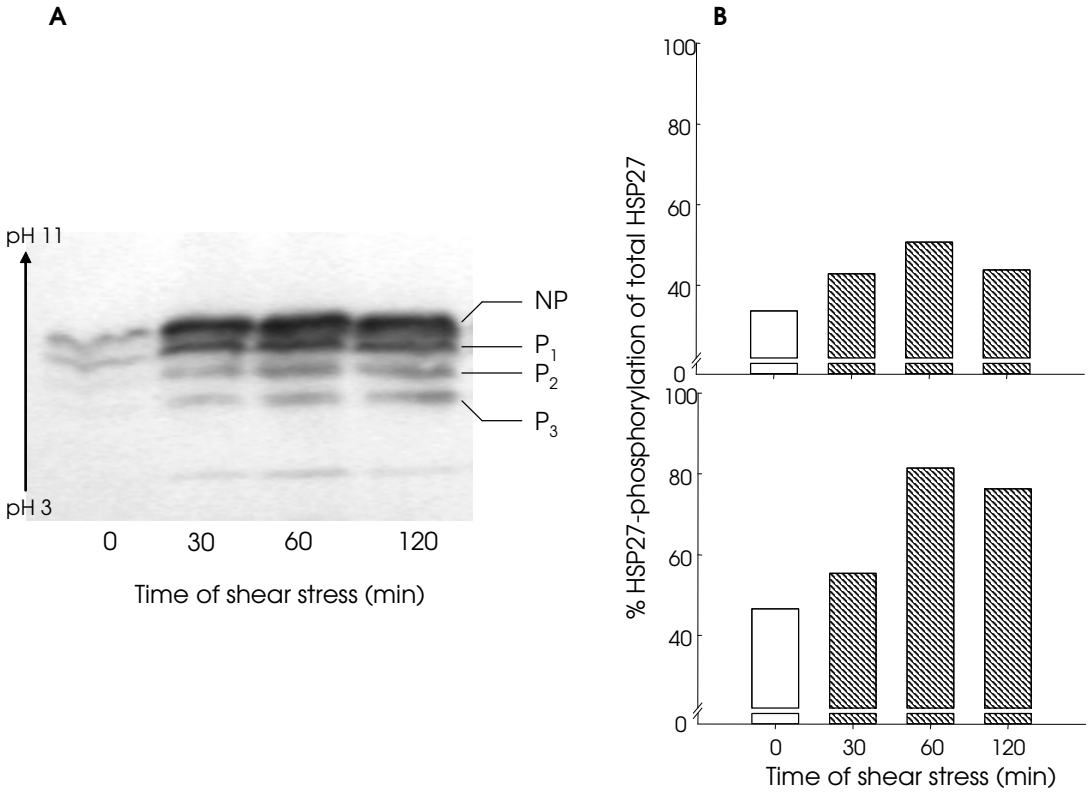


Figure 25. Isoelectric focusing for determining HSP27 phosphorylation after shear stress exposure. Shear stress exposure of endothelial cells seemed to increase the phosphorylation of HSP27 in a time dependent manner. PAEC was either kept as static control cells or exposed to shear stress (16 dyn/cm^2 for 30, 60 or 120 min). After treatment, the proteins were separated on a pH-gradient gel according to their isoelectric point (IpH). After separation, the proteins were blottet onto a nictrocellulose membrane and detected with a monoclonal mouse anti-HSP27 antibody. ***A.*** Shows a blot with the four different phosphorylation isoforms on a representative blot (NP: Non-phosphorylated, P_{1-3}: Phosphorylated). ***B.*** Shows the densitometric data expressed as the percentage of HSP27 phosphorylation of the total HSP27 after each time point from two independent experiments.

able to induce phosphorylation of HSP27 (figure 26 and 27). As shown in figure 25 the phosphorylation of HSP27 was significantly higher in endothelial cells treated with 0.5 U/ml compared to untreated control cells ($p<0.05$; n=5) when measured by PACE. Moreover, the phosphorylation did not increase further with a higher concentration of elastase (1.5 U/ml NS from 0 U/ml; n=5). The administration of 0.5 U/ml of elastase induced a 1.4 fold higher phosphorylation in treated cells compared to non-treated control cells ($p<0.05$; n=4), which was in the same range as the phosphorylation

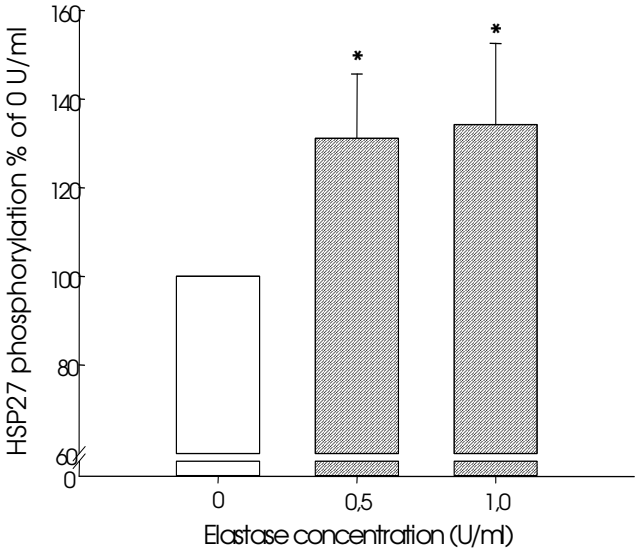


Figure 26. PACE analysis of HSP27 phosphorylation as a function of elastase concentration. Quantitative analysis of HSP27 phosphorylation by phosphospecific antibody cell-based ELISA (PACE), show HSP27 phosphorylation after elastase treatment. Porcine aortic endothelial cells seeded in a 96-well plate were either kept as untreated (0 U/ml elastase) or treated with different concentrations of porcine pancreatic elastase (0, 0.5 or 1.0 U/ml) for 2 hours. After treatment, the cells were permeabilized and the phosphorylation of HSP27 was detected by a phosphospecific HSP27 antibody. The bar graphs represent HSP27 phosphorylation expressed as % of the level in non-treated cells. *p<0.05 compared to untreated cells (0 U/ml). n=5.

caused by heat shock (42°C for 2 hours; n=4) measured by a self-made ELISA (figure 27A). Furthermore, the elastase induced HSP27 phosphorylation seemed to be reduced after integrin $\alpha_v\beta_3$ inhibition (Abxicimab (Abx)) (n=5; figure 27B).

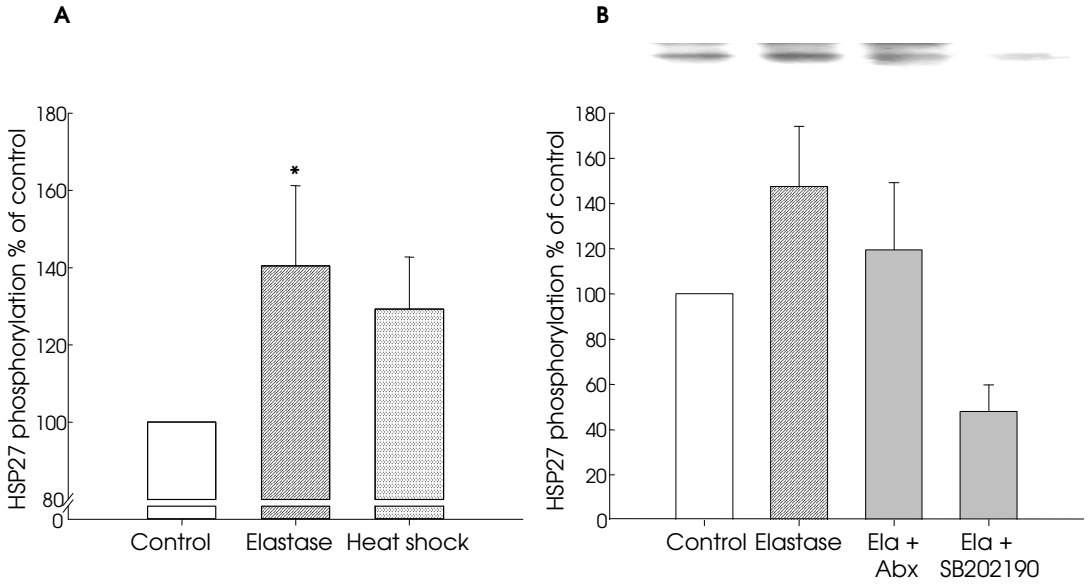


Figure 27. HSP27 phosphorylation after elastase treatment with 0.5 U/ml. *A*. Lysates from PAEC from either control cells, elastase-treated cells (0.5 U/ml, 2 hours) or heat shock-treated cells (42°C, 2 hours) were analyzed for heat shock protein 27 (HSP27) phosphorylation using a modified ELISA as described in the methods. The bar graphs represent HSP27 phosphorylation expressed as % of the level in non-treated cells. *p<0.05 compared to control. n=4. ***B***. Lysates from either untreated (control), elastase treated cells (0.5 U/ml, 2 hours), E + Abxicimab (Abx) (integrin $\alpha_v\beta_3$ inhibition) or E + SB202190 (p38 MAPK inhibition) were subjected to immuno-precipitation of phospho-serine. The precipitations were loaded in equal amounts on a 12% SDS-PAGE and probed with a polyclonal anti-HSP27 antibody as primary antibody. The upper panel shows a representative Western blot, whereas the lower panel shows the densitometric data expressed as % of the non-treated control level. Ela denotes elastase. n=5.

p38 MAP kinase activation

As mentioned in the introduction, HSP27 can be phosphorylated either via the p38 MAP kinase pathway or the p42/44 MAP kinase pathway. In the present study, specific inhibition of p38 MAP kinase caused a reduction in the HSP27 phosphorylation (n=5; figure 24B). To further assess for the role of p38 MAP kinase, Western blots were performed to investigate the phosphorylation of p38 - as well as p42/44 MAP kinase during elastase treatment. As seen in figure 27A, elastase treatment lead to a 1.8 fold higher p38 MAP kinase phosphorylation compared to non-treated control cells ($p<0.05$; n=4). In contrast, the p42/44 was not affected by the treatment (n=3; figure 28B). Furthermore, the phosphorylation of p38 MAP kinase was reduced by inhibition of integrin $\alpha_v\beta_3$ (n=4; figure 28A).

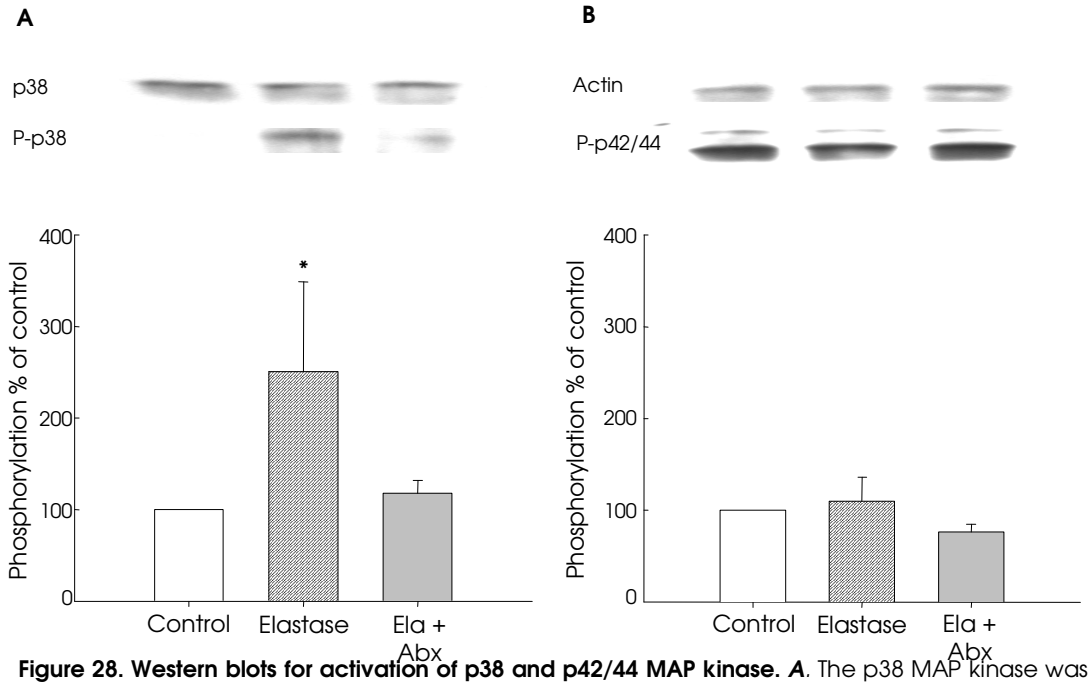
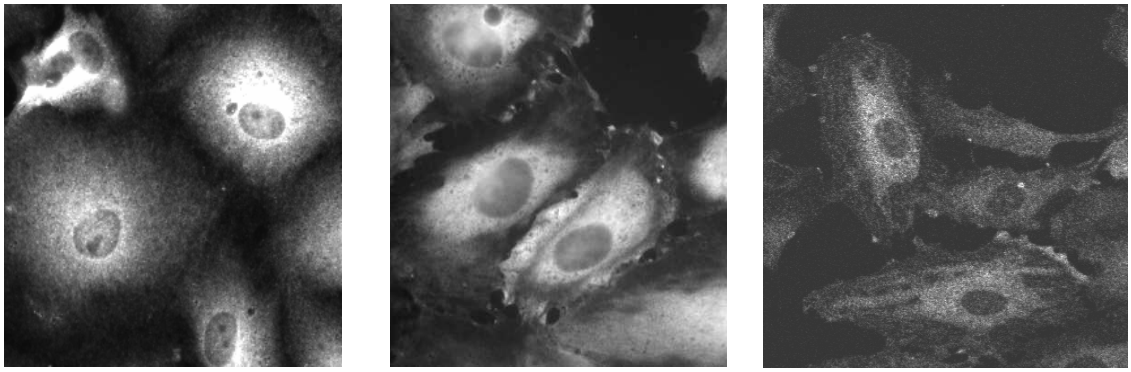


Figure 28. Western blots for activation of p38 and p42/44 MAP kinase. *A*. The p38 MAP kinase was phosphorylated in an integrin $\alpha_v\beta_3$ dependent manner after elastase treatment. *Upper panel* shows a representative Western blot. Lysates from cells from either untreated (control), elastase-treated (0.5 U/ml, 2 hours) or elastase + reopro (inhibitor of integrin $\alpha_v\beta_3$, 0.5 µg/ml) was separated on a 10 % SDS-PAGE, and immuno-blottet with a phospho-specific p38 MAP kinase antibody (lower bands). To ensure equal loading of protein, the membrane was re-probed with an anti-p38 antibody (upper bands). *Lower panel* shows the densitometric data (ration of phospho-p38 to p38) expressed as % of the non-treated control level. * p=0.05 compared to control. n=4. Ela denotes elastase. ***B***. The p42/44 MAP kinase was not phosphorylated after elastase treatment. The activation of the p42/44 is presented in a similar way as p38 activation (A). n=3.



Control Shear stress Elastase treatment

Figure 29. Immuno-histochemistry of HSP27. Subcellular localization of HSP27 in porcine aortic endothelial cells visualized by immuno-histochemistry show a translocation of HSP27 towards the plasma membrane upon shear stress exposure or elastase treatment. Direct immuno-fluorescence was performed on cells kept as non-treated control cells or cells exposed to shear stress (16 dyn/cm^2, 2 hours) or treated with porcine pancreatic elastase (0.5 U/ml, 2 hours). After treatment, cells were fixed, permeabilized and stained with a polyclonal rabbit anti-hsp27 antibody followed by incubation with a fluorescent-labeled anti-rabbit antibody. Representative pictures from 3 independent experiments.

HSP27 translocation

To assess the possibility that HSP27 is a carrier for bFGF to the cell membrane, immuno-staining of HSP27 in control cells, shear stress exposed or elastase treated cells was performed. Figure 28 shows representative immuno-histochemistry pictures of subcellular localization of HSP27 as it appear in 3 independent experiments. HSP27 displays a similar distribution pattern as bFGF (see figure 22), namely, after shear stress exposure as well as elastase treatment, HSP27, seemed to be localized in considerable amounts at the cell membrane.

In addition to immuno-histochemistry, measurements of the amount of HSP27 in membrane fractions of shear stress exposed- and elastase-treated cells together with their respective controls showed that significantly more HSP27 was found in the membrane fractions after the two treatments compared to their non-treated control cells (6-fold increase after shear stress, $p<0.05$ compared to control n= 3; and 2.5 fold increase after elastase treatment, $p<0.05$ compared to control, n=6; figure 30A and b, respectively). Moreover, inhibition of integrin $\alpha_v\beta_3$ (Abxicimab (Abx)) or p38

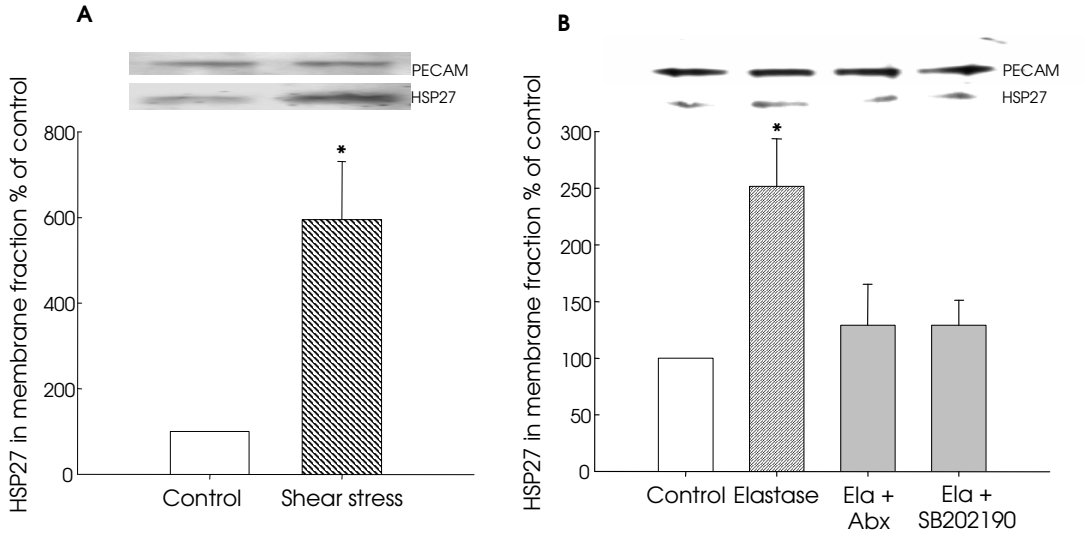


Figure 30. HSP27 in membrane fractions. Quantitative analysis of HSP27 translocation show increased HSP27 localization at the plasma membrane after shear stress exposure and elastase treatment. PAEC were kept either as controls, exposed to shear stress (16 dyn/cm^2, 2 hours) (A) or treated with porcine pancreatic elastase (0.5 U/ml, 2 hours) with or without an inhibitory antibody against integrin $\alpha_v\beta_3$ (Abxicimab (Abx), 0.5 µg/ml) or a specific inhibitor of p38 MAP kinase (SB202190, 1µM) (B). The membrane fractionation was done according to the description in the method section. The upper panel shows a representative Western blot. Ela denotes elastase. The bar graphs represent densitometric data (HSP27 / PECAM) expressed as % of the control level. *p<0.05 compared to control. n=3 (A). n=6 (B).

MAP kinase (SB202190) during elastase treatment resulted in a reduction in the amount of HSP27 in the membrane fraction (n=6; figure 30B).

HSP27 and bFGF co-precipitation

Co-precipitation of HSP27 and bFGF was determined by a self-made ELISA (see method section). First, the total amount of HSP27 (non-phosphorylated- plus phosphorylated-HSP27) was pulled down by an anti-HSP27 antibody. Second, only the phosphorylated-HSP27 was pulled down by a phospho-specific-HSP27 antibody. The amount of bFGF pulled down in both cases was detected by an anti-bFGF antibody. Lysate from control cells were either kept as native lysate or boiled (100°C, 5 min) to destroy the expected interaction between bFGF and HSP27 which thereby was used as a negative control for the interaction. An 8-fold higher amount of bFGF was pulled down together with the total amount of HSP27 in native lysate compared to boiled lysate (p<0.05; n=4; figure 31, dotted bars). Likewise, in the case where only the phosphorylated HSP27 was pulled down, an 8-fold higher amount of bFGF was

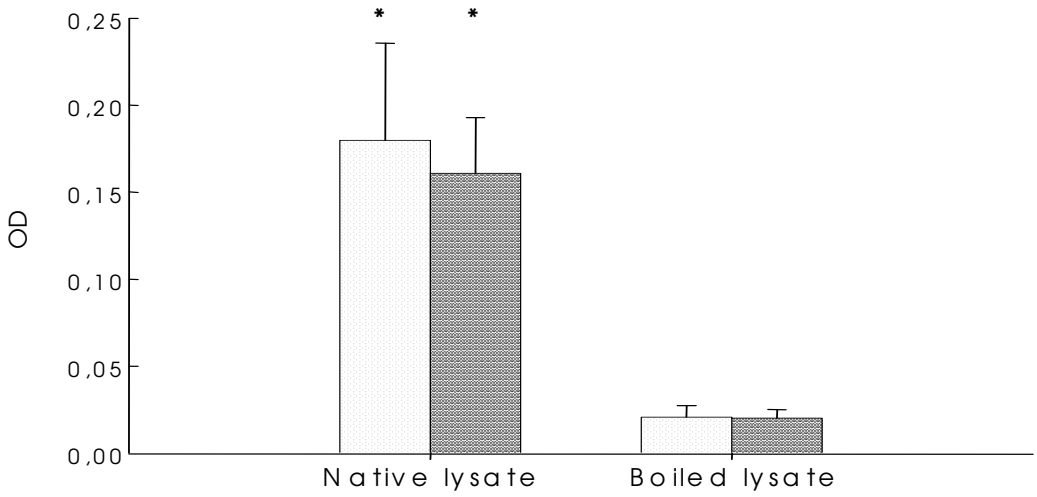


Figure 31. Co-precipitaion of phospho-HSP27 and bFGF. Lysates from non-treated PAEC were added to wells of a 96-well dish pre-coated with either a rabbit anti-HSP27 antibody (dotted bars) or a mouse anti-phospho-HSP27 antibody (squared bars). The total amount of HSP27 and the phosphorylated HSP27 proteins in the lysate were thereby pulled down, respectively. By using either a mouse anti-bFGF or a rabbit anti-bFGF antibody, the amount of bFGF pulled down together with the total HSP27 or the phospho-HSP27 was detected, respectively. The lysates were either boiled (100°C for 5 min) prior its application to the wells to destroy the protein-protein interactions or kept native, by which the interactions are preserved. *p<0.05 compared to pre-boiled lysate. n=3-4.

measured in the native lysate compared to boiled lysate ($p<0.05$; n=3; figure 31, squared bars).

Discussion

This study in cultured endothelial cells clearly showed that elastase, by interaction with the cellular matrix elicits a signaling cascade which initial step involves activation of integrin $\alpha_v\beta_3$. Through p38 MAP kinase signaling a phosphorylation of HSP27 is induced which is a prerequisite for the membrane translocation and finally release of bFGF from the intact cell. Further proof of concept the study suggests that shear stress of a magnitude that exists in small arteries and arterioles may well serve as a physiologic stimulus for the elastase induced bFGF release.

In the following, the results found in present study will be discussed in three parts. The first part deals with the role of elastase in bFGF release. The second part concerns the intracellular pathway leading to the release of bFGF and in the last part, the role of elastase in mechanotransduction of shear stress will be discussed.

bFGF release from endothelial cells

Elastase is a protease which has been shown to liberate bFGF from the extracellular matrix of endothelial cells. However, the data presented in this study provide evidence for the ability of elastase to induce an active release of endogenous bFGF. Moreover, the findings point to the fact that bFGF is released from the cells directly through the cell membrane.

Elastase as stimulus for bFGF release

To assess the possibility that elastase is involved in the bFGF release, conventional elastase was exogenously applied to static endothelial cells. The treatment with elastase led to a concentration dependent release of bFGF. A similar concentration dependent elastase-mediated release of bFGF was also demonstrated by Buczek-Thomas and Nugent (1999) in culture from lung pulmonary fibroblasts (Buczek-Thomas & Nugent, 1999). In that study the authors postulated that the elastase induced bFGF release originates from the ECM (Buczek-Thomas & Nugent, 1999; Rich *et al.*, 1996), where the bFGF is found bound to heparin sulfate proteoglycans (Yu *et al.*, 1993; Nugent & Iozzo, 2000). Buczek-Thomas and Nugent (1999) argued that elastase might be able to release bFGF by digesting proteoglycan core proteins and showed that the bFGF release correlated with release of sulfated glycosaminoglycans (GAG). They thereby concluded that the mechanism of elastase-mediated bFGF liberation is likely through disruption of matrix proteoglycans (Buczek-Thomas & Nugent, 1999). The view that bFGF is released by proteolytic digestion from the ECM is supported by other studies (Rich *et al.*, 1996; Saksela & Rifkin, 1990; Bashkin *et al.*, 1989; Ishai-Michaeli *et al.*, 1992; Whitelock *et al.*, 1996).

In contrast to these findings, I argue that the elastase treatment can lead to a direct release of endogenous bFGF. The argument for this comes from the finding that the release could be abolished by the inhibition of the integrin $\alpha_v\beta_3$. If the bFGF was solely released from the matrix, this release should not be affected by a cell-adhesion molecule. That bFGF indeed can be released from inside the cell and not only the matrix, has previously been demonstrated by Gloe *et al.* (2002) who

measured a decrease in the amount of bFGF within the cytoplasm after shear stress exposure of human umbilical vein endothelial cells (HUVEC).

One could argue that elastase in the concentration used here was lethal for the cells, since it is a degrading protein and could cause damage to the cells, which naturally would lead to an unspecific bFGF release. Cell damage as a mechanism of bFGF release was suggested by several groups (Hartnett *et al.*, 1999; Rhoads *et al.*, 2000; McNeil *et al.*, 1989; Brooks *et al.*, 1991). In contrast, it was in the present study demonstrated that cells treated with elastase up to a concentration of 0.5 U/ml did not show any sign of membrane damage and did not stain positive for propidium iodide indicating that they displayed an intact cell membrane. Furthermore, since the release could be reduced by inhibition of the integrin $\alpha_v\beta_3$, cell damage cannot be the reason for the observed bFGF release during elastase treatment. Rather, the release must be a regulated and controlled process, which previously has been concluded from other studies (Mignatti *et al.*, 1992; Florkiewicz *et al.*, 1995; Florkiewicz *et al.*, 1998; Gloe *et al.*, 2002; Backhaus *et al.*, 2004).

The release of bFGF

Even though the biological functions of bFGF are well characterized, only little is known about how bFGF is released from cells, since it lacks the signal sequence which is needed for classical protein secretion from the cell. In the present study, immuno-histochemistry pictures of bFGF could show that bFGF was localized at the cell membrane during elastase treatment in contrast to a more defuse subcellular distribution in control cells. This finding suggests that bFGF is released through the membrane. Further evidence for this statement comes from measurement of bFGF on the cell surface.

Basic FGF is a cytosolic protein normally not found on the cell surface. Measuring cell surface localization of a cytosolic protein is not commonly done and to my knowledge the present study is the first to determine cell surface localization of a cytosolic protein by FACE-technique. Furthermore, measuring bFGF on the cell surface by FACS was also a new idea and by setting up my own protocol I made it possible to quantify the cell surface localization of the protein. Thereby it was possible to show an elastase concentration-dependent cell surface localization of bFGF as

well as significantly more bFGF at the cell surface of elastase treated cells compared to non-treated control cells. That it is possible to measure bFGF on the cell outer surface means that the protein is being released from the cells directly through the cell membrane. The fact that bFGF can be detected by the antibody used in FACE and FACS is consistent with the view that the protein still is in the membrane but on its way out of the cell.

Alternatively to this view of how bFGF is being released, one study suggests a role of large granules in the bFGF secretion (Qu *et al.*, 1998), while others report of no apparent bFGF localization in vesicular structures (Engling *et al.*, 2002; Renko *et al.*, 1990). Other studies suggest bFGF to be released from cells via exocytosis (Piotrowicz *et al.*, 1997; Mignatti *et al.*, 1992) or membrane blebbing (Taverna *et al.*, 2003). However, if bFGF was released by exocytosis it would not be possible to detect the bFGF on the cell surface. The Na^+,K^+-ATPase is the only protein suggested to play a role in the export by forming a complex with bFGF and thereby catalyzing the transport of the growth factor out of the cell (Florkiewicz *et al.*, 1998). The ATPase could deliver a driving force for the transport of the relatively large bFGF molecule through the cell membrane. However, it remains to be studied further whether this mechanism played a role in our cell.

The conclusion that bFGF is released through the membrane is further supported by the studies showing that in CHO-cells GFP-labelled bFGF fused to dihydrofolate reductase (DHFR) is translocated across the cell membrane and thereby released in a folded conformation (Engling *et al.*, 2002; Backhaus *et al.*, 2004). This excludes another proposed transport patway, namely the ABC (ATP-binding cassette) transporter (Kuchler & Thorner, 1992), since ABC-transporter mediated protein export requires protein unfolding.

Heat shock proteins are proteins assisting protein folding and therefore, it might be speculated that they are able to assist bFGF through the membrane. In support of this idea, it has been shown that a heat shock protein, namely heat shock protein 27 (HSP27), facilitates the release of bFGF from endothelial cells (Piotrowicz *et. al* 1997). This is supported by the fact that the HSP27 intervene in the control of cell growth and differentiation (Horman *et al.*, 1999; Piotrowicz *et al.*, 1995), two processes also known to be mediated by bFGF (Nugent & Iozzo, 2000). Moreover, it would be a mechanism allowing bFGF to be released in its folded form. In the following part, the

role of HSP27 in bFGF release is discussed along with a possible intracellular signaling pathway.

The intracellular signaling cascade and bFGF release

Searching for a signaling pathway leading to bFGF release the present study provides evidence for that both bFGF as well as HSP27 were translocated towards the cell membrane upon stimulation. Moreover, both proteins were detected in higher amount at the cell membrane upon elastase treatment. Interestingly, bFGF could be co-precipitated with the phosphorylated HSP27 indicating that these proteins closely interact with each other.

Function of HSP27 in bFGF release

HSP27 as visualized by immuno-staining was found to be translocated to the cell membrane in elastase-treated cells similar to bFGF. Quantitative analysis of the HSP27 translocation by membrane fractionation confirmed that more HSP27 was found at the plasma membrane after elastase treatment. The observation that both proteins are translocated towards the membrane upon stimulation strongly suggests that HSP27 functions as a carrier for bFGF to the cell membrane, similar as it has been shown for HSP90 and eNOS (Ortiz *et al.*, 2004).

If this hypothesis is true, the two proteins must interact physically. Indeed, it could be shown that bFGF and HSP27 do interact, since bFGF could be pulled down together with HSP27. This was shown by a co-precipitating ELISA, which in the present study had to be established, since other co-precipitation assays were not appropriate in this case. It turned out that this ELISA was a more straight forward and more sensitive method than visualizing the co-precipitation by Western blotting. An interaction between HSP27 and bFGF has principally been shown before (Piotrowicz *et al.*, 1997), but the present study now shows for the first time that it specifically is the phosphorylated form of HSP27 which is interacting with bFGF. This is concluded from the fact that the amount bFGF pulled down together with the phosphorylated HSP27 was similar to the amount pulled down with the total (non-phospho- and phospho-

HSP27) HSP27. As control, boiled lysate was used, and showed negative results, since the protein-protein interaction in that case is destroyed.

This finding strongly indicates that HSP27 has to be phosphorylated in order to bind to bFGF and therefore, phosphorylation seems to a prerequisite for bFGF release. In fact, it could be shown that HSP27 was phosphorylated during elastase treatment. The importance of phospho-HSP27 is in accordance with a study showing that phosphorylated HSP27 in migrating endothelial cells in a wound healing assay was localized at the membrane of the leading edge (Piotrowicz *et al.*, 1998), which correlates with the localization of bFGF just in front of the migrating cells guiding the cells during migration (Nehls *et al.*, 1998).

HSP27 phosphorylation

In present study, the shear stress-mediated phosphorylation was determined by isoelectric focusing (IEF), which is a well documented method for determination of protein phosphorylation; measuring HSP27 phosphorylation by this method has previously been verified by ^{32}P-metabolic labeling (Li *et al.*, 1996; Landry *et al.*, 1992; Meier *et al.*, 2001; Larsen *et al.*, 1997). The reason for utilizing this technique and not Western blotting using a phospo-specific antibody was the lack of an appropriate phospho-HSP27 antibody. In IEF, the proteins are separated by charge, where phosphorylation of a protein is recognized as a shift in the mobility of the given protein. Therefore, no phospho-specific antibody had to be used. Most often the IEF is done in tube-gels; however, a self-established protocol for IEF in large slab gels was made, since the separation of the proteins was better and the handling of the gels easier. The disadvantage with the IEF is, however, that some contradictions exist in the literature regarding the isoelectric points (IpH) of the different phospho-isoforms of the HSP27. Therefore other approaches, such as self-made ELISA and PACE (see methods) were utilized in the present work to determine the HSP27 phosphorylation as a response to exogenous elastase treatment.

I was setting up my own ELISA and PACE, since at that time there was no commercial available ELISA- or PACE kit for phospho-HSP27. In these assays, a phospho-specific HSP27 antibody had to be used. As mentioned above, no phospho-HSP27 antibody was found to work in Western blotting; however, the

phospho-HSP27 antibody did work well in ELISA and PACE, which could be explained by the use of native proteins in these methods. By the self-made ELISA and PACE it was demonstrated that HSP27 phosphorylation was induced by shear stress as well as elastase treatment. The fact that other stimuli, such as shear stress (see below) and elastase as well as hypoxia and estrogen (Ishibashi *et al.*, 2001; Ishibashi *et al.*, 1995; Kuwabara *et al.*, 1995; Albuquerque *et al.*, 1998; Horman *et al.*, 1999), resulting in bFGF release simultaneously also lead b to HSP27 phosphorylation, supports the conclusion drawn from the present study, that HSP27 phosphorylation is prerequisite for bFGF release.

To be able to facilitate the release of bFGF from cells phospho-HSP27 and bFGF must be in the same place at the same time. Following HSP27 phosphorylation, some studies report that the protein is translocated to the nucleus in response to heat shock (Arrigo & Welch, 1987; Arrigo *et al.*, 1988) or ATP-depletion (Loktionova *et al.*, 1996), whereas other studies do not show any redistribution of HSP27 to the nucleus upon heat shock. In spite of the contradictory reports, a HSP27 redistribution to the nucleus upon phosphorylation could be a way for HSP27 and bFGF to meet each other, as high amounts of bFGF are localized in nucleus (Arnaud *et al.*, 1999; Quarto *et al.*, 1991; Florkiewicz *et al.*, 1991; Bugler *et al.*, 1991; Claus *et al.*, 2003). The latter was confirmed in the present study by immuno-staining. This finding fits very well to the studies stating that the high molecular weight (HMW) form of the bFGF is predominantly localized in the nucleus and the 18-kDa isoform not (Quarto *et al.*, 1991; Florkiewicz *et al.*, 1991; Bugler *et al.*, 1991), as well as that the HMW-bFGF is released in response to 17β-estradiol-stimulation (Piotrowicz *et al.*, 1997) – also a known stimulus for HSP27 phosphorylation (Piotrowicz *et al.*, 1995). However, the bFGF isoform released in this study has not been investigated.

HSP27 is either phosphorylated by the p38 MAPK pathway or the p42/44 MAPK pathway via MAPKAP kinase II (Rouse *et al.*, 1994; Stokoe *et al.*, 1992b) or by p38 MAPK via PRAK (New *et al.*, 1998). The data presented here provide substantial evidence for the involvement of p38 MAPK pathway, since the elastase-induced HSP27 phosphorylation was reduced by specific inhibition of p38 MAPK. The pathway was further dissected by demonstrating that elastase treatment led to p38 MAPK activation and not to p42/44 MAPK activation. This is in line with the finding that activation of integrin $\alpha_v\beta_3$ – being identified as a central player in the signaling

pathways studied here (will be discussed later) – does not affect the ERK1/2 (p42/44 MAPK) pathway (Weyts *et al.*, 2002). The explanation for the observed p38 MAPK and not p42/44 MAPK actiation could be that p38 MAPK activation is dependent upon specific integrin-matrix interactions, as demonstrated by Orr *et al.* (2005), whereas ERK1/2 activation does not seem to be dependent upon integrin-matrix interactions (Ponik & Pavalko, 2004; Weyts *et al.*, 2002) but is rather activated in non-focal-adhesion manner, such as receptor tyrosine kinases (Chen *et al.*, 1999), G-protein-coupled receptors (Gudi *et al.*, 1996), or ion channels (Olesen *et al.*, 1988).

Elastase in mechanotransduction

Shear stress-induced elastase activity

A significant involvement of elastase contribute to the shear stress-induced bFGF can be deduced from several observations. Firstly there was indeed a significantly higher elastase activity was measured in the medium after shear stress compared to their static controls at the same time as endothelial cells released bFGF. Secondary, the fact that shear stress-induced bFGF release could be prevented by inhibition of serine proteases certainly implies a role of serine proteases in the bFGF release. These two findings confirm that elastase takes part in the translation of the mechanical signal (shear stress) into a biochemical signal within the cells ending up with bFGF release. That proteases must play an important role in mechanotransduction is supported by the finding that mechano-stimulation such as shear stress and stretch leads to an enhanced activation and release of cysteine proteases and MMP-2 (Fukuda & Schmid-Schonbein, 2003; Rivilis *et al.*, 2002). Among different serine proteases I focused in the present study on the role of elastase in shear stress-induced bFGF release since yet unpublished results obtained from enzymograms, performed by other lab members, using different substrates like collagen, albumin and casein within the gel yielded no marked differences in proteolytic activity except for one digestive band at app. 20 kDa (the size of elastase). This band was much stronger after shear stress exposure compared to static cells, indicating that

elastase might be the main protease activated after shear stress (personal communication Gloe, T.).

Shear stress- vs. exogenous elastase-mediated bFGF release

The above discussed data suggest that the elastase is directly involved in the shear induced growth factor release. However, the exogenous elastase-mediated bFGF release was only 2-fold higher than control cells, which was less than the 10-fold increase in the release after shear stress, and additionally, the exogenous elastase concentration (0.5 U/ml) required to elicit a bFGF release was higher than the elastase concentration detected in the media after 2 hours of shear stress (0.015 U/ml). The explanation for these contradictories could be that shear stress makes the cells more sensitive to the elastase by deformation of the cell during shear stress turning on some intracellular pathways (Dalle-Donne *et al.*, 2001; Davies, 1995) which might enhance the cellular response to endogenous elastase. However, this does not seem to be the case, since shear stress and exogenously added elastase turn on a specific intracellular pathway involving integrin $\alpha_v\beta_3$, p38 MAPK activation and HSP27 phosphorylation leading to the release of bFGF.

Furthermore, bFGF has been demonstrated to be directly or indirectly interacting with the α-subunit of the Na^+,K^+-ATPase and the ATPase has been suggested to be involved in the bFGF release (Florkiewicz *et al.*, 1998). Hence, it might be possible that α-subunits of the Na^+,K^+-ATPase were translocated from intracellular storages to the plasma membrane upon shear stress to form more functional units of the ATPase in the membrane, as it has been observed in skeletal muscle cells as an acute response to exercise (Juel *et al.*, 2001).

Other explanations for the relatively low level of elastase measured in the medium after shear stress and at the same time a high bFGF release compared to exogenous elastase treatment simply might be that during shear stress, the elastase is released not only to the medium but also directly into the matrix leaving only a small amount of elastase in the medium. Therefore, the elastase activity measured in the medium during shear stress may be underestimated. At the same time, the elastase which is released directly into the matrix during shear stress is already at its place of action. In

contrast, the exogenously added elastase has to diffuse into the cell layer and into the matrix in order to place its action. This means that a higher concentration of exogenous elastase is needed to gain a similar effect as endogenous elastase. Possibly only a small amount of the exogenously added elastase reaches the matrix, which also would explain the lower bFGF release during exogenously added elastase.

Role of elastase in shear stress-induced bFGF release

The results discussed above suggest that elastase is involved in the mechanotransduction of shear stress into bFGF release, but do not indicate a possible mechanism. In the past, proteases have been considered to act mainly degradative. However, in recent years studies have shown that proteases are able to remodel the ECM and thereby exposing cryptic fragments that can lead to initiation of intracellular signaling (matricryptin) (Preston *et al.*, 2002; Geiger *et al.*, 2001; Mott & Werb, 2004; Faisal Khan *et al.*, 2002; Koshikawa *et al.*, 2000). Since the cells in the present study were primarily grown on laminin type I, and elastase is able to degrade this type of laminin into defined fragments (Kapur *et al.*, 2005) it is tempting to speculate that the released and/or activated elastase observed during shear stress is an important initiator of the mechanotransduction by remodeling the matrix. This view adds a new perspective to some already well established models of mechanotranduction. It should be mentioned that it is not yet clear how shear stress eliciting a release or activation of elastase. This remains to be determined in further experiments.

For many years it has been widely accepted that the transfer of shear stress forces first occurs at the luminal cell surface recognized by local displacement of sensors on the cell surface (mechanoreceptors) (Davies, 1995). These mechanosensors can be cell surface proteins (Davies, 1995) or distinct mechanosensitive ion channels (Sachs, 1988; Martinac *et al.*, 1987) such as potassium channels (Olesen *et al.*, 1988). It is believed that the stress activated mechanoreceptors transmit the mechanical signal into biochemical events by either one of two ways. One model suggests that the signal after being recognized at the luminal surface will be transferred into different part of the cell via second messengers (Davies, 1995). However, this model cannot

account for the transmission of shear stress into bFGF release, since this release was shown to be dependent upon the specific cell-adhesion molecule, integrin $\alpha_v\beta_3$ (Gloe *et al.*, 2002). A focal adhesion dependent mechnotransduction is in line with the second mechanotransduction model proposing that the recognized shear stress force at the luminal surface can be transmitted by deformation of the cytoskeleton to sites in the cell remote from the stimulus such as abluminal attachment sites which directly interact with integrins (Davies, 1995). There are, however, some contradictions between our results and the mentioned models of mechanotransduction: Davies (1995) and other authors (Chen *et al.*, 1999; Burridge *et al.*, 1988) suggest that the force is transmitted to the integrin from the luminal cell surface via the cytoskeleton, whereas the present study suggests that it is the shear stress-induced elastase activity and not the shear force directly that acts on the cell and thereby the integrin. The latter proposal is based upon the observations that, similar as for shear stress, also the exogenous elastase-mediated bFGF release in resting cells was prevented by inhibition of the integrin $\alpha_v\beta_3$.

The role of integrin $\alpha_v\beta_3$ in mechanotransduction

In both treatments, shear stress and elastase, the bFGF release was specifically dependent upon the integrin $\alpha_v\beta_3$, since inhibition of other integrins (mainly β_1 integrins) did not have any effect on the release (figure 20 and Gloe *et al.*, 2002), suggesting that a specific signaling pathway was turned on by the two types of treatment. This is supported by Tzima *et. al* (2001) who by immuno-staining with the anti-$\alpha_v\beta_3$ antibody specific for the activated integrin (WOW-1) demonstrated that shear stress specifically activates integrin $\alpha_v\beta_3$ (Tzima *et al.*, 2001). Moreover, the importance of this integrin in mechanotransduction in general is strengthened by the findings that pretreatment of endothelial cells by blocking anti-$\alpha_v\beta_3$ antibodies prevented the shear stress activation of ERK- and the JNK pathways as well as the IκB complex (Li *et al.*, 1997; Bhullar *et al.*, 1998).

That both shear stress- and exogenous elastase- induced bFGF release is critically dependent upon the same type of integrin does not only suggest that the release is mediated by a similar pathway but also that the release is critically dependent upon

cell-matrix interactions. The importance of the ECM in bFGF release mediated by elastase was demonstrated by measuring the cell surface localization of bFGF in elastase treated adherent and suspended cells. The localization of bFGF at the cell surface was taken as an indicator of bFGF translocation and subsequently release (as discussed above). Hence, that bFGF was measured at the cell surface after treatment of adherent cells but not of suspended cells indicates that elastase is not acting directly on the cells or their integrins but rather via the ECM. Taken into consideration that the signal from shear stress and elastase is transmitted to the cell through the same pathway, this observation is in agreement with the study showing that endothelial cells must be anchored to their matrix in order to sense and transduce signals in response to shear stress (Takahashi & Berk, 1996). This is supported by the finding by Jalali *et. al* 2001, who demonstrated that the shear stress induced mechanotransduction was abolished when new integrin-ECM ligand interactions were prevented (Jalali *et al.*, 2001). Furthermore, another study showed that estrogen-mediated bFGF release from endothelial cells was significantly higher when the cells were seeded on ECM proteins such as collagen IV, laminin type I and fibronectin as compared with collagen I or pure plastic (Albuquerque *et al.*, 1998). Moreover, it was demonstrated that whereas endothelial cells plated on fibronectin or fibrinogen respond to shear stress with an activation of NF-κB, cells grown on collagen or laminin did not (Orr *et al.*, 2005). All these mentioned studies emphasize the importance of the interplay between cells and their matrix in terms of cellular response to mechano stimulation.

Taken together, the mechanotransduction seems to be dependent upon integrin activation. It has been suggested that integrins can be activated either by cleavage, as shown for the α_{IIb} subunit of the platelet integrin $\alpha_{IIb}\beta_3$ (Si-Tahar *et al.*, 1997) or by phophorylation of the β_3 subunit of the integrin $\alpha_v\beta_3$ (Blystone *et al.*, 1996). However, clustering of the integrins into focal adhesion-like structures has also been proposed as an option for activation (Shyy & Chien, 2002; Giancotti & Ruoslahti, 1999; Avalos *et al.*, 2004; Weyts *et al.*, 2002), a process which can be established as integrins bind to the ECM (Giancotti & Ruoslahti, 1999; Shyy & Chien, 2002). However, since integrins constantly undergo binding turnover (Davies, 1995) the adhesion to the ECM by itself does not seem to be the signal, but rather the availability of new binding sites for the integrins might be essential for the integrin activation and the

subsequently initiation of intracellular signaling cascade (Tzima *et al.*, 2001; Kano *et al.*, 2000; Tzima *et al.*, 2002). New binding sites might be established by remodeling of the architectural fine structure within the ECM. Since elastase is able to produce fragments of laminin type I, the ECM protein primarily used in the present study, it is a possible candidate for activating integrins. Therefore, I propose that elastase, which is released and/or activated by shear stress exposure, cleaves the ECM and thereby liberates ECM fragtments or changes the matrix structure both of which are capable of activating the integrin $\alpha_v\beta_3$, presumably by changing their clustering. This integrin activation results in initiation of the specific intracellular signal cascade involving p38 MAPK activation and HSP27 phosphorylation and translocation, since these events are abolished by integrin $\alpha_v\beta_3$ inhibition. Subsequently, this leads to the release of bFGF from the endothelial cells.

Perspectives

Basic FGF is, as already pointed out, implicated in a variety of physiological processes, in pathogenesis of vascular diseases as well as in tumor growth. The bFGF acts primarily as a paracrine factor (Nugent & Iozzo, 2000; Valverius *et al.*, 1990) and hence, factors that lead to the release of bFGF from endothelial cells may not only facilitate tumor growth but also essential physiological processes such as development and angiogenesis. Hence, taking together, the knowledge about how bFGF is released may be very beneficial. The present study provides evidence for a role of elastase in mediating bFGF release from endothelial cells. Therefore, elastase and maybe other proteases, might be a key target in tumor growth as wells as a source to improve perfusion for example after myocardial ischemia. However, the organism is a complex system and thus, elastase may not only act on endothelial cells to release bFGF, but might also affect other proteins and systems. Hence, the interpretations of the role of elastase must be made with caution until its function has been verified *in vivo.*

With respect to physiologic remodelling processes, much interest has been focussed on shear stress induced vascular growth upon chronic increases in blood flow. It has been demonstrated that chronically decreased blood flow *in vivo* leads

to a reduction in the diameter of the artery (Langille & O'Donnell, 1986) and another study reports that the diameter is increased by chronically elevated blood flow (Tronc *et al.*, 1996). Furthermore, the increased expression of bFGF in smooth muscle cells from rabbits has been shown to correlate with flow induced arterial enlargement (Singh *et al.*, 1998). The present study and the study by Gloe *et. al* (2002) provides evidence for release of bFGF during shear stress *in vitro*. Exercise is a physiologic situation in which shear stress is elevated *in vivo*, and it has indeed been demonstrated that circulating bFGF is enhanced acutely after maximal exercise (Campuzano *et al.*, 2002) as well as chronically after 6 months of exercise training (Seida *et al.*, 2003). However, the relation between shear stress and bFGF *in vivo* is not entirely clear since other groups report of no change in exercise-induced circulating level bFGF (Adams *et al.*, 2004; Gu *et al.*, 2004) and again others observed even a decrease in the amount of bFGF within the first 10 min of exercise (Eliakim *et al.*, 2000) followed by a increase in the level of bFGF after 130 min of exercise (Nemet *et al.*, 2002). As it appears, inconsistencies exist regarding the bFGF respond to exercise, which could be explained by the fact that shear stress changes might have been different as they have not been really measured in most studies. Moreover, increased blood flow as a consequence of exercise not only affects the shear stress but also the pressure distribution along the vascular tree. Moreover, depending on the type of exercise, ischemia might also be a factor that influences bFGF release *in vivo*. Hence, the bFGF response is most likely dependent upon the type of exercise protocol utilized in the particular study, and it might therefore premature to associate increased flow *in vivo* always with increased bFGF release.

Angiogenesis is a second important focus with respect to bFGF effects *in vivo*. In fact, angiogenesis has been observed after 4 weeks of intensive one-legged exercise training, which correlated with an enhanced performance measured as the time to exhaustion during one-legged exercise (Jensen *et al.*, 2004). A role of bFGF in exercise- and hence shear stress-induced angiogenesis is suggested by Lederman *et. al* (2002) who demonstrated that intra-arterial administration of recombinant bFGF leads to angiogenesis and a significant increase in peak walking time after 90 days (Lederman *et al.*, 2002), supported by the finding that transendocardial administration of a recombinant gene coding for bFGF in patients with hibernating myocardium increased the perfusion and exercise tolerance (Kolomoets, 2001).

However, it is not generally accepted that bFGF is involved in initiation of angiogenesis (Brown *et al.*, 1998). Since bFGF can induce the expression of VEGF in endothelial cells, VEGF could decisively modulate the apparent angiogenic activity of bFGF (Seghezzi *et al.*, 1998).

Summary and conclusions

This study aimed to answer the question whether proteolytic remodeling of the extracellular matrix contributes to the shear stress-induced bFGF release. The results obtained in the present study present evidence for an important role of elastase as the protease which provides the link between the shear stress forces exerted on endothelial cells and the cellular response manifested as a release of bFGF. This

Table 7: Comparison of results obtained from shear stress – either in the present study or from the literature – with results from exogenously added elastase.

Treatment / Examining	Shear stress exposure (16 dyn/cm²)		Elastase treatment (0.5 U/ml)	
	Result	Figure / reference	Result	Figure / reference
bFGF release	5 fold increase	Fig. 16	2 fold increase	Fig. 19
+ $\alpha_v\beta_3$ inhibition	Reduction	Gloe *et al. 2002*	Reduction	Fig. 20
bFGF cell surface localization	Increase	Fig. 21	1.5 fold increase	Fig. 23A
+ $\alpha_v\beta_3$ inhibition			Reduction	Fig. 23A
HSP27 in membrane fraction (translocation)	6 fold increase	Fig. 29A	2.5 fold increase	Fig. 29B
+ $\alpha_v\beta_3$ inhibition			Reduced	Fig. 29B
HSP27 phosphorylation	Increase	Fig. 24	1.4 fold increase	Fig. 25+26A
+ $\alpha_v\beta_3$ inhibition	Reduction	Gloe *et al* 2002	Reduction	Fig. 26B
+ p38 inhibition	Reduction	Azuma *et al.*2001	Reduction	Fig. 26B
p38 phosphorylation	Increase	Azuma *et al.*2001	2.8 fold increase	Fig. 27A
+ $\alpha_v\beta_3$ inhibition			Reduction	Fig. 27A

conclusion is based on the observations that shear stress and exogenously added elastase initiate the same subcellular signaling pathway (table 7). The present study reveals the following chain of events: Shear stress releases and/or activates elastase, which subsequently remodels the extracellular matrix. It remains to be clarified whether this occurs either by clustering the integrins as a consequence of altered matrix structure or by releasing a fragment in the matrix (matricryptin) that specifically stimulates $\alpha_v\beta_3$. The activation of the integrin initiates an intracellular signaling cascade involving phosphorylation of HSP27 via the p38 MAP kinase pathway. The phosphorylation of HSP27 is necessary for its interaction with bFGF. The translocation of this complex to the membrane is then a prerequisite for the release of bFGF which is not possible through the classical seretion pathway. A summary of the pathways initiated by shear stress and clarified in this study is schematically illustrated in figure 32.

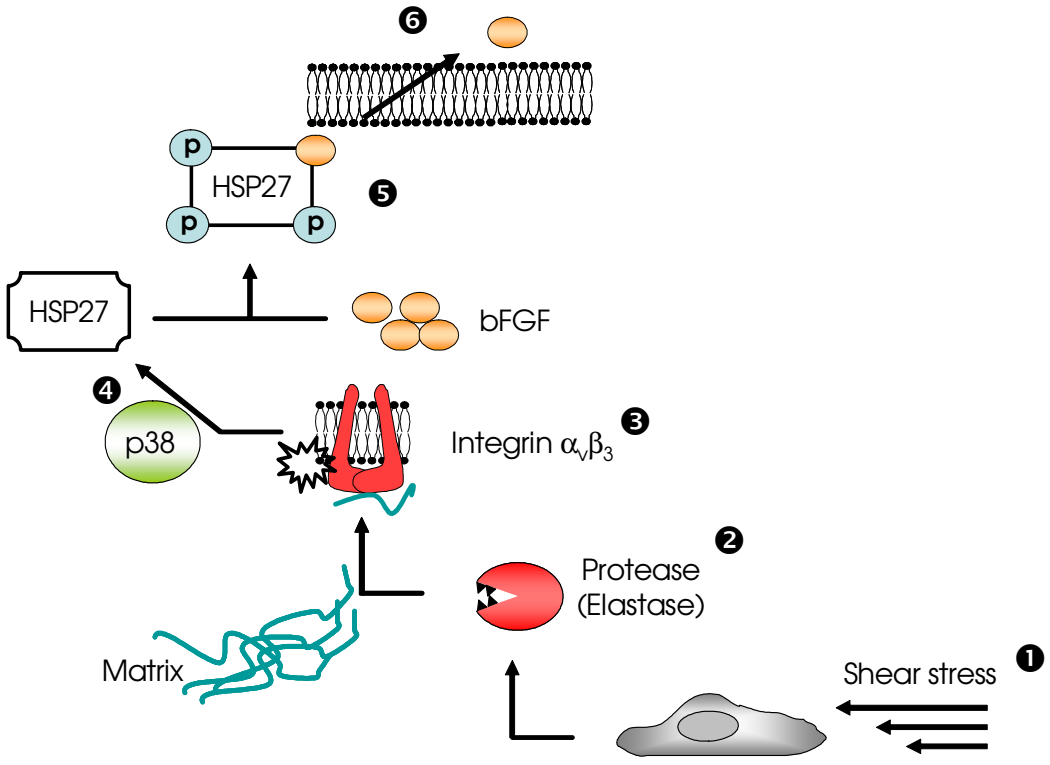


Figure 32. Proposed signaling pathway for shear stress-induced bFGF release: Shear stress forces on endothelial cells (1) induce an enhanced elastase activity (2). The elastase can activate integrin $\alpha_v\beta_3$ via the matrix (3) leading to p38 MAPK activation and subsequently phosphorylation of heat shock protein 27 (HSP27) (4). Following phosphorylation, HSP27 binds bFGF and translocate towards the plasma membrane (5), through where bFGF is released form the endothelial cells (6).

References

Abraham, J. A., Mergia, A., Whang, J. L., Tumolo, A., Friedman, J., Hjerrild, K. A., Gospodarowicz, D., & Fiddes, J. C. (1986a). Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. *Science* **233**, 545-548.

Abraham, J. A., Whang, J. L., Tumolo, A., Mergia, A., Friedman, J., Gospodarowicz, D., & Fiddes, J. C. (1986b). Human basic fibroblast growth factor: nucleotide sequence and genomic organization. *EMBO J.* **5**, 2523-2528.

Acevedo, A. D., Bowser, S. S., Gerritsen, M. E., & Bizios, R. (1993). Morphological and proliferative responses of endothelial cells to hydrostatic pressure: role of fibroblast growth factor. *J.Cell Physiol* **157**, 603-614.

Adams, V., Lenk, K., Linke, A., Lenz, D., Erbs, S., Sandri, M., Tarnok, A., Gielen, S., Emmrich, F., Schuler, G., & Hambrecht, R. (2004). Increase of circulating endothelial progenitor cells in patients with coronary artery disease after exercise-induced ischemia. *Arterioscler.Thromb.Vasc.Biol.* **24**, 684-690.

Alberts, B., Johnson, A., Lewis, j., Raff, M., Roberts, K., & Walter, P. (2002a). How cells read the genome: From DNA to protein. In *Molecular biology of the cell* pp. 299-374. Garland Publisher, New York.

Alberts, B., Johnson, A., Lewis, j., Raff, M., Roberts, K., & Walter, P. (2002b). Intracellular compartments and protein sorting. In *Molecular Biology of the Cell* pp. 659-710. Garland Publisher, New York.

Alberts, B., Johnson, A., Lewis, j., Raff, M., Roberts, K., & Walter, P. (2002c). Intracellular vesicular traffic. In *Molecular Biology of the Cell* pp. 711-766. Garland Publisher, New York.

Albuquerque, M. L., Akiyama, S. K., & Schnaper, H. W. (1998). Basic fibroblast growth factor release by human coronary artery endothelial cells is enhanced by matrix proteins, 17beta-estradiol, and a PKC signaling pathway. *Exp.Cell Res.* **245**, 163-169.

Ambalavanan, N., Bulger, A., & Philips III, J. B. (1999). Hypoxia-induced release of peptide growth factors from neonatal porcine pulmonary artery smooth muscle cells. *Biol.Neonate* **76**, 311-319.

Andrei, C., Dazzi, C., Lotti, L., Torrisi, M. R., Chimini, G., & Rubartelli, A. (1999). The secretory route of the leaderless protein interleukin 1beta involves exocytosis of endolysosome-related vesicles. *Mol.Biol.Cell* **10**, 1463-1475.

Arnaud, E., Touriol, C., Boutonnet, C., Gensac, M. C., Vagner, S., Prats, H., & Prats, A. C. (1999). A new 34-kilodalton isoform of human fibroblast growth factor 2 is cap dependently synthesized by using a non-AUG start codon and behaves as a survival factor. *Mol.Cell Biol.* **19**, 505-514.

Arrigo, A. P., Suhan, J. P., & Welch, W. J. (1988). Dynamic changes in the structure and intracellular locale of the mammalian low-molecular-weight heat shock protein. *Mol.Cell Biol.* **8**, 5059-5071.

Arrigo, A. P. & Welch, W. J. (1987). Characterization and purification of the small 28,000-dalton mammalian heat shock protein. *J.Biol.Chem.* **262**, 15359-15369.

Asahara, T., Bauters, C., Zheng, L. P., Takeshita, S., Bunting, S., Ferrara, N., Symes, J. F., & Isner, J. M. (1995). Synergistic effect of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in vivo. *Circulation* **92**, II365-II371.

Avalos, A. M., Arthur, W. T., Schneider, P., Quest, A. F., Burridge, K., & Leyton, L. (2004). Aggregation of integrins and RhoA activation are required for Thy-1-induced morphological changes in astrocytes. *J.Biol.Chem.* **279**, 39139-39145.

Azuma, N., Akasaka, N., Kito, H., Ikeda, M., Gahtan, V., Sasajima, T., & Sumpio, B. E. (2001). Role of p38 MAP kinase in endothelial cell alignment induced by fluid shear stress. *Am.J.Physiol Heart Circ.Physiol* **280**, H189-H197.

Azuma, N., Duzgun, S. A., Ikeda, M., Kito, H., Akasaka, N., Sasajima, T., & Sumpio, B. E. (2000). Endothelial cell response to different mechanical forces. *J.Vasc.Surg.* **32**, 789-794.

Backhaus, R., Zehe, C., Wegehingel, S., Kehlenbach, A., Schwappach, B., & Nickel, W. (2004). Unconventional protein secretion: membrane translocation of FGF-2 does not require protein unfolding. *J.Cell Sci.* **117**, 1727-1736.

Barchowsky, A., Williams, M. E., Benz, C. C., & Chepenik, K. P. (1994). Oxidant-sensitive protein phosphorylation in endothelial cells. *Free Radic.Biol.Med.* **16**, 771-777.

Bashkin, P., Doctrow, S., Klagsbrun, M., Svahn, C. M., Folkman, J., & Vlodavsky, I. (1989). Basic fibroblast growth factor binds to subendothelial extracellular matrix and is released by heparitinase and heparin-like molecules. *Biochemistry* **28**, 1737-1743.

Ben Levy, R., Hooper, S., Wilson, R., Paterson, H. F., & Marshall, C. J. (1998). Nuclear export of the stress-activated protein kinase p38 mediated by its substrate MAPKAP kinase-2. *Curr.Biol.* **8**, 1049-1057.

Benndorf, R., Hayess, K., Ryazantsev, S., Wieske, M., Behlke, J., & Lutsch, G. (1994). Phosphorylation and supramolecular organization of murine small heat shock protein HSP25 abolish its actin polymerization-inhibiting activity. *J.Biol.Chem.* **269**, 20780-20784.

Berk, B. C., Corson, M. A., Peterson, T. E., & Tseng, H. (1995). Protein kinases as mediators of fluid shear stress stimulated signal transduction in endothelial cells: a hypothesis for calcium-dependent and calcium-independent events activated by flow. *J.Biomech.* **28**, 1439-1450.

Bhullar, I. S., Li, Y. S., Miao, H., Zandi, E., Kim, M., Shyy, J. Y., & Chien, S. (1998). Fluid shear stress activation of IkappaB kinase is integrin-dependent. *J.Biol.Chem.* **273**, 30544-30549.

Bikfalvi, A., Klein, S., Pintucci, G., & Rifkin, D. B. (1997). Biological roles of fibroblast growth factor-2. *Endocr.Rev.* **18**, 26-45.

Blystone, S. D., Lindberg, F. P., Williams, M. P., McHugh, K. P., & Brown, E. J. (1996). Inducible tyrosine phosphorylation of the beta3 integrin requires the alphaV integrin cytoplasmic tail. *J.Biol.Chem.* **271**, 31458-31462.

Brooks, R. A., Burrin, J. M., & Kohner, E. M. (1991). Characterization of release of basic fibroblast growth factor from bovine retinal endothelial cells in monolayer cultures. *Biochem.J.* **276 (Pt 1)**, 113-120.

Brown, M. D., Hudlicka, O., Damon, D., & Duling, B. R. (1996). Vasoactive effects of basic and acidic fibroblast growth factors in hamster cheek pouch arterioles. *Int.J.Microcirc.Clin.Exp.* **16**, 308-312.

Brown, M. D., Walter, H., Hansen-Smith, F. M., Hudlicka, O., & Egginton, S. (1998). Lack of involvement of basic fibroblast growth factor (FGF-2) in capillary growth in skeletal muscles exposed to long-term contractile activity. *Angiogenesis.* **2**, 81-91.

Bryant, S. R., Bjercke, R. J., Erichsen, D. A., Rege, A., & Lindner, V. (1999). Vascular remodeling in response to altered blood flow is mediated by fibroblast growth factor-2. *Circ.Res.* **84**, 323-328.

Buczek-Thomas, J. A. & Nugent, M. A. (1999). Elastase-mediated release of heparan sulfate proteoglycans from pulmonary fibroblast cultures. A mechanism for basic fibroblast growth factor (bFGF) release and attenuation of bfgf binding following elastase-induced injury. *J.Biol.Chem.* **274**, 25167-25172.

Bugler, B., Amalric, F., & Prats, H. (1991). Alternative initiation of translation determines cytoplasmic or nuclear localization of basic fibroblast growth factor. *Mol.Cell Biol.* **11**, 573-577.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G., & Turner, C. (1988). Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu.Rev.Cell Biol.* **4**, 487-525.

Campuzano, R., Barrios, V., Cuevas, B., Asin-Cardiel, E., Muela, A., Castro, J. M., Fernandez-Ayerdi, A., & Cuevas, P. (2002). Serum basic fibroblast growth factor levels in exercise-induced myocardial ischemia more likely a marker of endothelial dysfunction than a marker of ischemia? *Eur.J.Med.Res.* **7**, 93-97.

Chaturvedi, K. & Sarkar, D. K. (2004). Involvement of protein kinase C-dependent mitogen-activated protein kinase p44/42 signaling pathway for cross-talk between estradiol and transforming growth factor-beta3 in increasing basic fibroblast growth factor in folliculostellate cells. *Endocrinology* **145**, 706-715.

Chen, C. H., Poucher, S. M., Lu, J., & Henry, P. D. (2004). Fibroblast growth factor 2: from laboratory evidence to clinical application. *Curr.Vasc.Pharmacol.* **2**, 33-43.

Chen, K. D., Li, Y. S., Kim, M., Li, S., Yuan, S., Chien, S., & Shyy, J. Y. (1999). Mechanotransduction in response to shear stress. Roles of receptor tyrosine kinases, integrins, and Shc. *J.Biol.Chem.* **274**, 18393-18400.

Chen, R. H., Sarnecki, C., & Blenis, J. (1992). Nuclear localization and regulation of erk- and rsk-encoded protein kinases. *Mol.Cell Biol.* **12**, 915-927.

Claus, P., Doring, F., Gringel, S., Muller-Ostermeyer, F., Fuhlrott, J., Kraft, T., & Grothe, C. (2003). Differential intranuclear localization of fibroblast growth factor-2 isoforms and specific interaction with the survival of motoneuron protein. *J.Biol.Chem.* **278**, 479-485.

Clifton, A. D., Young, P. R., & Cohen, P. (1996). A comparison of the substrate specificity of MAPKAP kinase-2 and MAPKAP kinase-3 and their activation by cytokines and cellular stress. *FEBS Lett.* **392**, 209-214.

Coso, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T., & Gutkind, J. S. (1995). The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell* **81**, 1137-1146.

Cozzolino, F., Torcia, M., Lucibello, M., Morbidelli, L., Ziche, M., Platt, J., Fabiani, S., Brett, J., & Stern, D. (1993). Interferon-alpha and interleukin 2 synergistically enhance basic fibroblast growth factor synthesis and induce release, promoting endothelial cell growth. *J.Clin.Invest* **91**, 2504-2512.

Cucina, A., Borrelli, V., Di Carlo, A., Pagliei, S., Corvino, V., Santoro-D'Angelo, L., Cavallaro, A., & Sterpetti, A. V. (1999a). Thrombin induces production of growth factors from aortic smooth muscle cells. *J.Surg.Res.* **82**, 61-66.

Cucina, A., Corvino, V., Sapienza, P., Borrelli, V., Lucarelli, M., Scarpa, S., Strom, R., Santoro-D'Angelo, L., & Cavallaro, A. (1999b). Nicotine regulates basic fibroblastic growth factor and transforming growth factor beta1 production in endothelial cells. *Biochem.Biophys.Res.Commun.* **257**, 306-312.

Dalle-Donne, I., Rossi, R., Milzani, A., Di Simplicio, P., & Colombo, R. (2001). The actin cytoskeleton response to oxidants: from small heat shock protein phosphorylation to changes in the redox state of actin itself. *Free Radic.Biol.Med.* **31**, 1624-1632.

Davies, P. F. (1995). Flow-mediated endothelial mechanotransduction. *Physiol Rev.* **75**, 519-560.

Davies, P. F., Barbee, K. A., Volin, M. V., Robotewskyj, A., Chen, J., Joseph, L., Griem, M. L., Wernick, M. N., Jacobs, E., Polacek, D. C., dePaola, N., & Barakat, A. I. (1997). Spatial relationships in early signaling events of flow-mediated endothelial mechanotransduction. *Annu.Rev.Physiol* **59**, 527-549.

Davies, P. F., Robotewskyj, A., & Griem, M. L. (1993). Endothelial cell adhesion in real time. Measurements in vitro by tandem scanning confocal image analysis. *J.Clin.Invest* **91**, 2640-2652.

Davies, P. F., Robotewskyj, A., & Griem, M. L. (1994). Quantitative studies of endothelial cell adhesion. Directional remodeling of focal adhesion sites in response to flow forces. *J.Clin.Invest* **93**, 2031-2038.

Dono, R., Texido, G., Dussel, R., Ehmke, H., & Zeller, R. (1998). Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice. *EMBO J.* **17**, 4213-4225.

Dow, J. K. & deVere White, R. W. (2000). Fibroblast growth factor 2: its structure and property, paracrine function, tumor angiogenesis, and prostate-related mitogenic and oncogenic functions. *Urology* **55**, 800-806.

Dulbecco, R. & Freeman, G. (1959). Plaque production by polyoma virus. *Virology* **8**, 396-397.

Eliakim, A., Oh, Y., & Cooper, D. M. (2000). Effect of single wrist exercise on fibroblast growth factor-2, insulin-like growth factor, and growth hormone. *Am.J.Physiol Regul.Integr.Comp Physiol* **279**, R548-R553.

Engel, K., Kotlyarov, A., & Gaestel, M. (1998). Leptomycin B-sensitive nuclear export of MAPKAP kinase 2 is regulated by phosphorylation. *EMBO J.* **17**, 3363-3371.

Engling, A., Backhaus, R., Stegmayer, C., Zehe, C., Seelenmeyer, C., Kehlenbach, A., Schwappach, B., Wegehingel, S., & Nickel, W. (2002). Biosynthetic FGF-2 is targeted to non-lipid raft microdomains following translocation to the extracellular surface of CHO cells. *J.Cell Sci.* **115**, 3619-3631.

Faisal Khan, K. M., Laurie, G. W., McCaffrey, T. A., & Falcone, D. J. (2002). Exposure of cryptic domains in the alpha 1-chain of laminin-1 by elastase stimulates macrophages urokinase and matrix metalloproteinase-9 expression. *J.Biol.Chem.* **277**, 13778-13786.

Ferrara, N. (2000). VEGF: an update on biological and therapeutic aspects. *Curr.Opin.Biotechnol.* **11**, 617-624.

Florkiewicz, R. Z., Anchin, J., & Baird, A. (1998). The inhibition of fibroblast growth factor-2 export by cardenolides implies a novel function for the catalytic subunit of Na+,K+-ATPase. *J.Biol.Chem.* **273**, 544-551.

Florkiewicz, R. Z., Baird, A., & Gonzalez, A. M. (1991). Multiple forms of bFGF: differential nuclear and cell surface localization. *Growth Factors* **4**, 265-275.

Florkiewicz, R. Z., Majack, R. A., Buechler, R. D., & Florkiewicz, E. (1995). Quantitative export of FGF-2 occurs through an alternative, energy- dependent, non-ER/Golgi pathway. *J.Cell Physiol* **162**, 388-399.

Florkiewicz, R. Z. & Sommer, A. (1989). Human basic fibroblast growth factor gene encodes four polypeptides: three initiate translation from non-AUG codons. *Proc.Natl.Acad.Sci.U.S.A* **86**, 3978-3981.

Frangos, J. A., Eskin, S. G., McIntire, L. V., & Ives, C. L. (1985). Flow effects on prostacyclin production by cultured human endothelial cells. *Science* **227**, 1477-1479.

Frangos, J. A., McIntire, L. V., & Eskin, S. G. (1987). Shear stress induced stimulation of mammalian cell metabolism. *Biotechnology and Bioengineering* **32**, 1053-1060.

Freshney, N. W., Rawlinson, L., Guesdon, F., Jones, E., Cowley, S., Hsuan, J., & Saklatvala, J. (1994). Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. *Cell* **78**, 1039-1049.

Fukuda, S. & Schmid-Schonbein, G. W. (2003). Regulation of CD18 expression on neutrophils in response to fluid shear stress. *Proc.Natl.Acad.Sci.U.S.A* **100**, 13152-13157.

Gaitanaki, C., Konstantina, S., Chrysa, S., & Beis, I. (2003). Oxidative stress stimulates multiple MAPK signalling pathways and phosphorylation of the small HSP27 in the perfused amphibian heart. *J.Exp.Biol.* **206**, 2759-2769.

Geiger, B., Bershadsky, A., Pankov, R., & Yamada, K. M. (2001). Transmembrane crosstalk between the extracellular matrix--cytoskeleton crosstalk. *Nat.Rev.Mol.Cell Biol.* **2**, 793-805.

Giancotti, F. G. & Ruoslahti, E. (1999). Integrin signaling. *Science* **285**, 1028-1032.

Gloe, T. & Pohl, U. (2002). Laminin binding conveys mechanosensing in endothelial cells. *News Physiol Sci.* **17**, 166-169.

Gloe, T., Sohn, H. Y., Meininger, G. A., & Pohl, U. (2002). Shear stress-induced release of basic fibroblast growth factor from endothelial cells is mediated by matrix interaction via integrin alpha(v)beta3. *J.Biol.Chem.* **277**, 23453-23458.

Gospodarowicz, D. (1974). Localisation of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth. *Nature* **249**, 123-127.

Gospodarowicz, D. (1975). Purification of a fibroblast growth factor from bovine pituitary. *J.Biol.Chem.* **250**, 2515-2520.

Gospodarowicz, D., Jones, K. L., & Sato, G. (1974). Purification of a growth factor for ovarian cells from bovine pituitary glands. *Proc.Natl.Acad.Sci.U.S.A* **71**, 2295-2299.

Gu, J. W., Gadonski, G., Wang, J., Makey, I., & Adair, T. H. (2004). Exercise increases endostatin in circulation of healthy volunteers. *BMC.Physiol* **4**, 2.

Guay, J., Lambert, H., Gingras-Breton, G., Lavoie, J. N., Huot, J., & Landry, J. (1997). Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27. *J.Cell Sci.* **110 (Pt 3)**, 357-368.

Gudi, S. R., Clark, C. B., & Frangos, J. A. (1996). Fluid flow rapidly activates G proteins in human endothelial cells. Involvement of G proteins in mechanochemical signal transduction. *Circ.Res.* **79**, 834-839.

Guesdon, F., Freshney, N., Waller, R. J., Rawlinson, L., & Saklatvala, J. (1993). Interleukin 1 and tumor necrosis factor stimulate two novel protein kinases that phosphorylate the heat shock protein hsp27 and beta-casein. *J.Biol.Chem.* **268**, 4236-4243.

Hamon, Y., Luciani, M. F., Becq, F., Verrier, B., Rubartelli, A., & Chimini, G. (1997). Interleukin-1beta secretion is impaired by inhibitors of the Atp binding cassette transporter, ABC1. *Blood* **90**, 2911-2915.

Hartnett, M. E., Garcia, C. M., & D'Amore, P. A. (1999). Release of bFGF, an endothelial cell survival factor, by osmotic shock. *Invest Ophthalmol.Vis.Sci.* **40**, 2945-2951.

Hedges, J. C., Dechert, M. A., Yamboliev, I. A., Martin, J. L., Hickey, E., Weber, L. A., & Gerthoffer, W. T. (1999). A role for p38(MAPK)/HSP27 pathway in smooth muscle cell migration. *J.Biol.Chem.* **274**, 24211-24219.

Hentges, S. & Sarkar, D. K. (2001). Transforming growth factor-beta regulation of estradiol-induced prolactinomas. *Front Neuroendocrinol.* **22**, 340-363.

Herbert, J. M., Dupuy, E., Laplace, M. C., Zini, J. M., Bar, S. R., & Tobelem, G. (1994). Thrombin induces endothelial cell growth via both a proteolytic and a non-proteolytic pathway. *Biochem.J.* **303 (Pt 1)**, 227-231.

Horman, S., Fokan, D., Mosselmans, R., Mairesse, N., & Galand, P. (1999). Anti-sense inhibition of small-heat-shock-protein (HSP27) expression in MCF-7 mammary-carcinoma cells induces their spontaneous acquisition of a secretory phenotype. *Int.J.Cancer* **82**, 574-582.

Huang, A., Sun, D., Carroll, M. A., Jiang, H., Smith, C. J., Connetta, J. A., Falck, J. R., Shesely, E. G., Koller, A., & Kaley, G. (2001). EDHF mediates flow-induced dilation in skeletal muscle arterioles of female eNOS-KO mice. *Am.J.Physiol Heart Circ.Physiol* **280**, H2462-H2469.

Hughes, R. C. (1999). Secretion of the galectin family of mammalian carbohydrate-binding proteins. *Biochim.Biophys.Acta* **1473**, 172-185.

Huot, J., Lambert, H., Lavoie, J. N., Guimond, A., Houle, F., & Landry, J. (1995). Characterization of 45-kDa/54-kDa HSP27 kinase, a stress-sensitive kinase which may activate the phosphorylation-dependent protective function of mammalian 27-kDa heat-shock protein HSP27. *Eur.J.Biochem.* **227**, 416-427.

Ishai-Michaeli, R., Svahn, C. M., Weber, M., Chajek-Shaul, T., Korner, G., Ekre, H. P., & Vlodavsky, I. (1992). Importance of size and sulfation of heparin in release of basic fibroblast growth factor from the vascular endothelium and extracellular matrix. *Biochemistry* **31**, 2080-2088.

Ishibashi, H., Nakagawa, K., Nakashima, Y., & Sueishi, K. (1995). Conditioned media of carcinoma cells cultured in hypoxic microenvironment stimulate angiogenesis in vitro; relationship to basic fibroblast growth factor. *Virchows Arch.* **425**, 561-568.

Ishibashi, H., Shiratuchi, T., Nakagawa, K., Onimaru, M., Sugiura, T., Sueishi, K., & Shirasuna, K. (2001). Hypoxia-induced angiogenesis of cultured human salivary gland carcinoma cells enhances vascular endothelial growth factor production and basic fibroblast growth factor release. *Oral Oncol.* **37**, 77-83.

Jackson, A., Friedman, S., Zhan, X., Engleka, K. A., Forough, R., & Maciag, T. (1992). Heat shock induces the release of fibroblast growth factor 1 from NIH 3T3 cells. *Proc.Natl.Acad.Sci.U.S.A* **89**, 10691-10695.

Jackson, A., Tarantini, F., Gamble, S., Friedman, S., & Maciag, T. (1995). The release of fibroblast growth factor-1 from NIH 3T3 cells in response to temperature involves the function of cysteine residues. *J.Biol.Chem.* **270**, 33-36.

Jalali, S., del Pozo, M. A., Chen, K., Miao, H., Li, Y., Schwartz, M. A., Shyy, J. Y., & Chien, S. (2001). Integrin-mediated mechanotransduction requires its dynamic interaction with specific extracellular matrix (ECM) ligands. *Proc.Natl.Acad.Sci.U.S.A* **98**, 1042-1046.

Jaye, M., Schlessinger, J., & Dionne, C. A. (1992). Fibroblast growth factor receptor tyrosine kinases: molecular analysis and signal transduction. *Biochim.Biophys.Acta* **1135**, 185-199.

Jensen, L., Bangsbo, J., & Hellsten, Y. (2004). Effect of high intensity training on capillarization and presence of angiogenic factors in human skeletal muscle. *J.Physiol* **557**, 571-582.

Jo, H., Sipos, K., Go, Y. M., Law, R., Rong, J., & McDonald, J. M. (1997). Differential effect of shear stress on extracellular signal-regulated kinase and N-terminal Jun kinase in endothelial cells. Gi2- and Gbeta/gamma-dependent signaling pathways. *J.Biol.Chem.* **272**, 1395-1401.

Juel, C., Grunnet, L., Holse, M., Kenworthy, S., Sommer, V., & Wulff, T. (2001). Reversibility of exercise-induced translocation of Na+-K+ pump subunits to the plasma membrane in rat skeletal muscle. *Pflugers Arch.* **443**, 212-217.

Kano, Y., Katoh, K., & Fujiwara, K. (2000). Lateral zone of cell-cell adhesion as the major fluid shear stress-related signal transduction site. *Circ.Res.* **86**, 425-433.

Kapur, S., Chen, S. T., Baylink, D. J., & Lau, K. H. (2004). Extracellular signal-regulated kinase-1 and -2 are both essential for the shear stress-induced human osteoblast proliferation. *Bone* **35**, 525-534.

Kapur, S., Mohan, S., Baylink, D. J., & Lau, K. H. (2005). Fluid shear stress synergizes with IGF-I on osteoblast proliferation through integrin-dependent activation of IGF-I mitogenic signaling pathway. *J.Biol.Chem.*

Kato, K., Hasegawa, K., Goto, S., & Inaguma, Y. (1994). Dissociation as a result of phosphorylation of an aggregated form of the small stress protein, hsp27. *J.Biol.Chem.* **269**, 11274-11278.

Kolomoets, N. M. (2001). (Endothelial dysfunction and its clinical significance (new trend in cardiology)). *Voen.Med.Zh.* **322**, 29-35, 96.

Koshikawa, N., Giannelli, G., Cirulli, V., Miyazaki, K., & Quaranta, V. (2000). Role of cell surface metalloprotease MT1-MMP in epithelial cell migration over laminin-5. *J.Cell Biol.* **148**, 615-624.

Kuchler, K. & Thorner, J. (1992). Secretion of peptides and proteins lacking hydrophobic signal sequences: the role of adenosine triphosphate-driven membrane translocators. *Endocr.Rev.* **13**, 499-514.

Kuwabara, K., Ogawa, S., Matsumoto, M., Koga, S., Clauss, M., Pinsky, D. J., Lyn, P., Leavy, J., Witte, L., Joseph-Silverstein, J., & . (1995). Hypoxia-mediated induction of acidic/basic fibroblast growth factor and platelet-derived growth factor in mononuclear phagocytes stimulates growth of hypoxic endothelial cells. *Proc.Natl.Acad.Sci.U.S.A* **92**, 4606-4610.

Landry, J., Lambert, H., Zhou, M., Lavoie, J. N., Hickey, E., Weber, L. A., & Anderson, C. W. (1992). Human HSP27 is phosphorylated at serines 78 and 82 by heat shock and mitogen-activated kinases that recognize the same amino acid motif as S6 kinase II. *J.Biol.Chem.* **267**, 794-803.

Langille, B. L. & O'Donnell, F. (1986). Reductions in arterial diameter produced by chronic decreases in blood flow are endothelium-dependent. *Science* **231**, 405-407.

Larsen, J. K., Yamboliev, I. A., Weber, L. A., & Gerthoffer, W. T. (1997). Phosphorylation of the 27-kDa heat shock protein via p38 MAP kinase and MAPKAP kinase in smooth muscle. *Am.J.Physiol* **273**, L930-L940.

Lavoie, J. N., Lambert, H., Hickey, E., Weber, L. A., & Landry, J. (1995). Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27. *Mol.Cell Biol.* **15**, 505-516.

Lazarous, D. F., Scheinowitz, M., Shou, M., Hodge, E., Rajanayagam, S., Hunsberger, S., Robison, W. G., Jr., Stiber, J. A., Correa, R., Epstein, S. E., & . (1995). Effects of chronic systemic administration of basic fibroblast growth factor on collateral development in the canine heart. *Circulation* **91**, 145-153.

Lederman, R. J., Mendelsohn, F. O., Anderson, R. D., Saucedo, J. F., Tenaglia, A. N., Hermiller, J. B., Hillegass, W. B., Rocha-Singh, K., Moon, T. E., Whitehouse, M. J., & Annex, B. H. (2002). Therapeutic angiogenesis with recombinant fibroblast growth factor-2 for intermittent claudication (the TRAFFIC study): a randomised trial. *Lancet* **359**, 2053-2058.

Lee, P. L., Johnson, D. E., Cousens, L. S., Fried, V. A., & Williams, L. T. (1989). Purification and complementary DNA cloning of a receptor for basic fibroblast growth factor. *Science* **245**, 57-60.

LEIBOVITZ, A. (1963). THE GROWTH AND MAINTENANCE OF TISSUE-CELL CULTURES IN FREE GAS EXCHANGE WITH THE ATMOSPHERE. *Am.J.Hyg.* **78**, 173-180.

Lenormand, P., Sardet, C., Pages, G., L'Allemain, G., Brunet, A., & Pouyssegur, J. (1993). Growth factors induce nuclear translocation of MAP kinases (p42mapk and p44mapk) but not of their activator MAP kinase kinase (p45mapkk) in fibroblasts. *J.Cell Biol.* **122**, 1079-1088.

Lepidi, S., Sterpetti, A. V., Cucina, A., Di Carlo, A., Patrizi, A. L., Palumbo, R., Bernucci, P., Santoro-D'Angelo, L., & Cavallaro, A. (1995). bFGF release is dependent on flow conditions in experimental vein grafts. *Eur.J.Vasc.Endovasc.Surg.* **10**, 450-458.

Levin, E. G. & Santell, L. (1991). Thrombin- and histamine-induced signal transduction in human endothelial cells. Stimulation and agonist-dependent desensitization of protein phosphorylation. *J.Biol.Chem.* **266**, 174-181.

Li, S., Chen, B. P., Azuma, N., Hu, Y. L., Wu, S. Z., Sumpio, B. E., Shyy, J. Y., & Chien, S. (1999). Distinct roles for the small GTPases Cdc42 and Rho in endothelial responses to shear stress. *J.Clin.Invest* **103**, 1141-1150.

Li, S., Kim, M., Hu, Y. L., Jalali, S., Schlaepfer, D. D., Hunter, T., Chien, S., & Shyy, J. Y. (1997). Fluid shear stress activation of focal adhesion kinase. Linking to mitogen-activated protein kinases. *J.Biol.Chem.* **272**, 30455-30462.

Li, S., Piotrowicz, R. S., Levin, E. G., Shyy, Y. J., & Chien, S. (1996). Fluid shear stress induces the phosphorylation of small heat shock proteins in vascular endothelial cells. *Am.J.Physiol* **271**, C994-1000.

Li, Y., Zheng, J., Bird, I. M., & Magness, R. R. (2004). Mechanisms of shear stress-induced endothelial nitric-oxide synthase phosphorylation and expression in ovine fetoplacental artery endothelial cells. *Biol.Reprod.* **70**, 785-796.

Liekens, S., De Clercq, E., & Neyts, J. (2001). Angiogenesis: regulators and clinical applications. *Biochem.Pharmacol.* **61**, 253-270.

Loktionova, S. A., Ilyinskaya, O. P., Gabai, V. L., & Kabakov, A. E. (1996). Distinct effects of heat shock and ATP depletion on distribution and isoform patterns of human Hsp27 in endothelial cells. *FEBS Lett.* **392**, 100-104.

Lowry, O. H., ROSEBROUGH, N. J., FARR, A. L., & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J.Biol.Chem.* **193**, 265-275.

Martinac, B., Buechner, M., Delcour, A. H., Adler, J., & Kung, C. (1987). Pressure-sensitive ion channel in Escherichia coli. *Proc.Natl.Acad.Sci.U.S.A* **84**, 2297-2301.

Mazzag, B. M., Tamaresis, J. S., & Barakat, A. I. (2003). A model for shear stress sensing and transmission in vascular endothelial cells. *Biophys.J.* **84**, 4087-4101.

McLaughlin, M. M., Kumar, S., McDonnell, P. C., Van Horn, S., Lee, J. C., Livi, G. P., & Young, P. R. (1996). Identification of mitogen-activated protein (MAP) kinase-activated protein kinase-3, a novel substrate of CSBP p38 MAP kinase. *J.Biol.Chem.* **271**, 8488-8492.

McNeil, P. L., Muthukrishnan, L., Warder, E., & D'Amore, P. A. (1989). Growth factors are released by mechanically wounded endothelial cells. *J.Cell Biol.* **109**, 811-822.

Meier, M., King, G. L., Clermont, A., Perez, A., Hayashi, M., & Feener, E. P. (2001). Angiotensin AT(1) receptor stimulates heat shock protein 27 phosphorylation in vitro and in vivo. *Hypertension* **38**, 1260-1265.

Mignatti, P., Morimoto, T., & Rifkin, D. B. (1991). Basic fibroblast growth factor released by single, isolated cells stimulates their migration in an autocrine manner. *Proc.Natl.Acad.Sci.U.S.A* **88**, 11007-11011.

Mignatti, P., Morimoto, T., & Rifkin, D. B. (1992). Basic fibroblast growth factor, a protein devoid of secretory signal sequence, is released by cells via a pathway independent of the endoplasmic reticulum-Golgi complex. *J.Cell Physiol* **151**, 81-93.

Miller, D. L., Ortega, S., Bashayan, O., Basch, R., & Basilico, C. (2000). Compensation by fibroblast growth factor 1 (FGF1) does not account for the mild phenotypic defects observed in FGF2 null mice. *Mol.Cell Biol.* **20**, 2260-2268.

Minden, A., Lin, A., Claret, F. X., Abo, A., & Karin, M. (1995). Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell* **81**, 1147-1157.

Miron, T., Vancompernolle, K., Vandekerckhove, J., Wilchek, M., & Geiger, B. (1991). A 25-kD inhibitor of actin polymerization is a low molecular mass heat shock protein. *J.Cell Biol.* **114**, 255-261.

Miron, T., Wilchek, M., & Geiger, B. (1988). Characterization of an inhibitor of actin polymerization in vinculin-rich fraction of turkey gizzard smooth muscle. *Eur.J.Biochem.* **178**, 543-553.

Mott, J. D. & Werb, Z. (2004). Regulation of matrix biology by matrix metalloproteinases. *Curr.Opin.Cell Biol.* **16**, 558-564.

Nehls, V., Herrmann, R., & Huhnken, M. (1998). Guided migration as a novel mechanism of capillary network remodeling is regulated by basic fibroblast growth factor. *Histochem.Cell Biol.* **109**, 319-329.

Nemet, D., Hong, S., Mills, P. J., Ziegler, M. G., Hill, M., & Cooper, D. M. (2002). Systemic vs. local cytokine and leukocyte responses to unilateral wrist flexion exercise. *J.Appl.Physiol* **93**, 546-554.

New, L., Jiang, Y., Zhao, M., Liu, K., Zhu, W., Flood, L. J., Kato, Y., Parry, G. C., & Han, J. (1998). PRAK, a novel protein kinase regulated by the p38 MAP kinase. *EMBO J.* **17**, 3372-3384.

Ni, H., Wang, X. S., Diener, K., & Yao, Z. (1998). MAPKAPK5, a novel mitogen-activated protein kinase (MAPK)-activated protein kinase, is a substrate of the extracellular-regulated kinase (ERK) and p38 kinase. *Biochem.Biophys.Res.Commun.* **243**, 492-496.

Nickel, W. (2003). The mystery of nonclassical protein secretion. A current view on cargo proteins and potential export routes. *Eur.J.Biochem.* **270**, 2109-2119.

Nugent, M. A. & Iozzo, R. V. (2000). Fibroblast growth factor-2. *Int.J.Biochem.Cell Biol.* **32**, 115-120.

Okada-Ban, M., Thiery, J. P., & Jouanneau, J. (2000). Fibroblast growth factor-2. *Int.J.Biochem.Cell Biol.* **32**, 263-267.

Olesen, S. P., Clapham, D. E., & Davies, P. F. (1988). Haemodynamic shear stress activates a K+ current in vascular endothelial cells. *Nature* **331**, 168-170.

Ornitz, D. M. & Itoh, N. (2001). Fibroblast growth factors. *Genome Biol.* **2**, REVIEWS3005.

Ornitz, D. M., Xu, J., Colvin, J. S., McEwen, D. G., MacArthur, C. A., Coulier, F., Gao, G., & Goldfarb, M. (1996). Receptor specificity of the fibroblast growth factor family. *J.Biol.Chem.* **271**, 15292-15297.

Orr, A. W., Sanders, J. M., Bevard, M., Coleman, E., Sarembock, I. J., & Schwartz, M. A. (2005). The subendothelial extracellular matrix modulates NF-kappaB activation by flow: a potential role in atherosclerosis. *J.Cell Biol.* **169**, 191-202.

Ortega, S., Ittmann, M., Tsang, S. H., Ehrlich, M., & Basilico, C. (1998). Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. *Proc.Natl.Acad.Sci.U.S.A* **95**, 5672-5677.

Ortiz, P. A., Hong, N. J., & Garvin, J. L. (2004). Luminal flow induces eNOS activation and translocation in the rat thick ascending limb. II. Role of PI3-kinase and Hsp90. *Am.J.Physiol Renal Physiol* **287**, F281-F288.

Pampori, N., Hato, T., Stupack, D. G., Aidoudi, S., Cheresh, D. A., Nemerow, G. R., & Shattil, S. J. (1999). Mechanisms and consequences of affinity modulation of integrin alpha(V)beta(3) detected with a novel patch-engineered monovalent ligand. *J.Biol.Chem.* **274**, 21609-21616.

Papetti, M. & Herman, I. M. (2002). Mechanisms of normal and tumor-derived angiogenesis. *Am.J.Physiol Cell Physiol* **282**, C947-C970.

Pintucci, G., Moscatelli, D., Saponara, F., Biernacki, P. R., Baumann, F. G., Bizekis, C., Galloway, A. C., Basilico, C., & Mignatti, P. (2002). Lack of ERK activation and cell migration in FGF-2-deficient endothelial cells. *FASEB J.* **16**, 598-600.

Piotrowicz, R. S., Hickey, E., & Levin, E. G. (1998). Heat shock protein 27 kDa expression and phosphorylation regulates endothelial cell migration. *FASEB J.* **12**, 1481-1490.

Piotrowicz, R. S., Martin, J. L., Dillman, W. H., & Levin, E. G. (1997). The 27-kDa heat shock protein facilitates basic fibroblast growth factor release from endothelial cells. *J.Biol.Chem.* **272**, 7042-7047.

Piotrowicz, R. S., Weber, L. A., Hickey, E., & Levin, E. G. (1995). Accelerated growth and senescence of arterial endothelial cells expressing the small molecular weight heat-shock protein HSP27. *FASEB J.* **9**, 1079-1084.

Ploegh, H. L. (1995). Electrophoresis. In *Current protocol in protein science*, eds. Coligan, J. E., Dunn, B. M., Speicher, E. W., & Wingfield, P. T., John Wiley & Sons, Inc.I.

Pohl, U., Holtz, J., Busse, R., & Bassenge, E. (1986). Crucial role of endothelium in the vasodilator response to increased flow in vivo. *Hypertension* **8**, 37-44.

Ponik, S. M. & Pavalko, F. M. (2004). Formation of focal adhesions on fibronectin promotes fluid shear stress induction of COX-2 and PGE2 release in MC3T3-E1 osteoblasts. *J.Appl.Physiol* **97**, 135-142.

Preston, G. A., Zarella, C. S., Pendergraft, W. F., III, Rudolph, E. H., Yang, J. J., Sekura, S. B., Jennette, J. C., & Falk, R. J. (2002). Novel effects of neutrophil-derived proteinase 3 and elastase on the vascular endothelium involve in vivo cleavage of NF-kappaB and proapoptotic changes in JNK, ERK, and p38 MAPK signaling pathways. *J.Am.Soc.Nephrol.* **13**, 2840-2849.

Prior, B. M., Yang, H. T., & Terjung, R. L. (2004). What makes vessels grow with exercise training? *J.Appl.Physiol* **97**, 1119-1128.

Qu, Z., Kayton, R. J., Ahmadi, P., Liebler, J. M., Powers, M. R., Planck, S. R., & Rosenbaum, J. T. (1998). Ultrastructural immunolocalization of basic fibroblast growth factor in mast cell secretory granules. Morphological evidence for bfgf release through degranulation. *J.Histochem.Cytochem.* **46**, 1119-1128.

Quarto, N., Finger, F. P., & Rifkin, D. B. (1991). The NH2-terminal extension of high molecular weight bFGF is a nuclear targeting signal. *J.Cell Physiol* **147**, 311-318.

Rajanayagam, M. A., Shou, M., Thirumurti, V., Lazarous, D. F., Quyyumi, A. A., Goncalves, L., Stiber, J., Epstein, S. E., & Unger, E. F. (2000). Intracoronary basic fibroblast growth factor enhances myocardial collateral perfusion in dogs. *J.Am.Coll.Cardiol.* **35**, 519-526.

Renko, M., Quarto, N., Morimoto, T., & Rifkin, D. B. (1990). Nuclear and cytoplasmic localization of different basic fibroblast growth factor species. *J.Cell Physiol* **144**, 108-114.

Resnick, N., Yahav, H., Shay-Salit, A., Shushy, M., Schubert, S., Zilberman, L. C., & Wofovitz, E. (2003). Fluid shear stress and the vascular endothelium: for better and for worse. *Prog.Biophys.Mol.Biol.* **81**, 177-199.

Rhoads, D. N., Eskin, S. G., & McIntire, L. V. (2000). Fluid flow releases fibroblast growth factor-2 from human aortic smooth muscle cells. *Arterioscler.Thromb.Vasc.Biol.* **20**, 416-421.

Rich, C. B., Nugent, M. A., Stone, P., & Foster, J. A. (1996). Elastase release of basic fibroblast growth factor in pulmonary fibroblast cultures results in down-regulation of elastin gene transcription. A role for basic fibroblast growth factor in regulating lung repair. *J.Biol.Chem.* **271**, 23043-23048.

Rivilis, I., Milkiewicz, M., Boyd, P., Goldstein, J., Brown, M. D., Egginton, S., Hansen, F. M., Hudlicka, O., & Haas, T. L. (2002). Differential involvement of MMP-2 and VEGF during muscle stretch- versus shear stress-induced angiogenesis. *Am.J.Physiol Heart Circ.Physiol* **283**, H1430-H1438.

Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T., & Nebreda, A. R. (1994). A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell* **78**, 1027-1037.

Rousseau, S., Houle, F., Landry, J., & Huot, J. (1997). p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. *Oncogene* **15**, 2169-2177.

Rubartelli, A., Cozzolino, F., Talio, M., & Sitia, R. (1990). A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. *EMBO J.* **9**, 1503-1510.

Sachs, F. (1988). Mechanical transduction in biological systems. *Crit Rev.Biomed.Eng* **16**, 141-169.

Saksela, O. & Rifkin, D. B. (1990). Release of basic fibroblast growth factor-heparan sulfate complexes from endothelial cells by plasminogen activator-mediated proteolytic activity. *J.Cell Biol.* **110**, 767-775.

Samaniego, F., Markham, P. D., Gallo, R. C., & Ensoli, B. (1995). Inflammatory cytokines induce AIDS-Kaposi's sarcoma-derived spindle cells to produce and release basic fibroblast growth factor and enhance Kaposi's sarcoma-like lesion formation in nude mice. *J.Immunol.* **154**, 3582-3592.

Samaniego, F., Markham, P. D., Gendelman, R., Gallo, R. C., & Ensoli, B. (1997). Inflammatory cytokines induce endothelial cells to produce and release basic fibroblast growth factor and to promote Kaposi's sarcoma-like lesions in nude mice. *J.Immunol.* **158**, 1887-1894.

Seghezzi, G., Patel, S., Ren, C. J., Gualandris, A., Pintucci, G., Robbins, E. S., Shapiro, R. L., Galloway, A. C., Rifkin, D. B., & Mignatti, P. (1998). Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis. *J.Cell Biol.* **141**, 1659-1673.

Seida, A., Wada, J., Kunitomi, M., Tsuchiyama, Y., Miyatake, N., Fujii, M., Kira, S., Takahashi, K., Shikata, K., & Makino, H. (2003). Serum bFGF levels are reduced in

Japanese overweight men and restored by a 6-month exercise education. *Int.J.Obes.Relat Metab Disord.* **27**, 1325-1331.

Seternes, O. M., Johansen, B., Hegge, B., Johannessen, M., Keyse, S. M., & Moens, U. (2002). Both binding and activation of p38 mitogen-activated protein kinase (MAPK) play essential roles in regulation of the nucleocytoplasmic distribution of MAPK-activated protein kinase 5 by cellular stress. *Mol.Cell Biol.* **22**, 6931-6945.

Shibata, F., Baird, A., & Florkiewicz, R. Z. (1991). Functional characterization of the human basic fibroblast growth factor gene promoter. *Growth Factors* **4**, 277-287.

Shyy, J. Y. & Chien, S. (1997). Role of integrins in cellular responses to mechanical stress and adhesion. *Curr.Opin.Cell Biol.* **9**, 707-713.

Shyy, J. Y. & Chien, S. (2002). Role of integrins in endothelial mechanosensing of shear stress. *Circ.Res.* **91**, 769-775.

Si-Tahar, M., Pidard, D., Balloy, V., Moniatte, M., Kieffer, N., Van Dorsselaer, A., & Chignard, M. (1997). Human neutrophil elastase proteolytically activates the platelet integrin alphaIIbbeta3 through cleavage of the carboxyl terminus of the alphaIIb subunit heavy chain. Involvement in the potentiation of platelet aggregation. *J.Biol.Chem.* **272**, 11636-11647.

Singh, T. M., Abe, K. Y., Sasaki, T., Zhuang, Y. J., Masuda, H., & Zarins, C. K. (1998). Basic fibroblast growth factor expression precedes flow-induced arterial enlargement. *J.Surg.Res.* **77**, 165-173.

Stokoe, D., Campbell, D. G., Nakielny, S., Hidaka, H., Leevers, S. J., Marshall, C., & Cohen, P. (1992a). MAPKAP kinase-2; a novel protein kinase activated by mitogen-activated protein kinase. *EMBO J.* **11**, 3985-3994.

Stokoe, D., Engel, K., Campbell, D. G., Cohen, P., & Gaestel, M. (1992b). Identification of MAPKAP kinase 2 as a major enzyme responsible for the phosphorylation of the small mammalian heat shock proteins. *FEBS Lett.* **313**, 307-313.

Takahashi, M. & Berk, B. C. (1996). Mitogen-activated protein kinase (ERK1/2) activation by shear stress and adhesion in endothelial cells. Essential role for a herbimycin-sensitive kinase. *J.Clin.Invest* **98**, 2623-2631.

Taverna, S., Ghersi, G., Ginestra, A., Rigogliuso, S., Pecorella, S., Alaimo, G., Saladino, F., Dolo, V., Dell'Era, P., Pavan, A., Pizzolanti, G., Mignatti, P., Presta, M., & Vittorelli, M.

L. (2003). Shedding of membrane vesicles mediates fibroblast growth factor-2 release from cells. *J.Biol.Chem.* **278**, 51911-51919.

Towbin, H., Staehelin, T., & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc.Natl.Acad.Sci.U.S.A* **76**, 4350-4354.

Tronc, F., Wassef, M., Esposito, B., Henrion, D., Glagov, S., & Tedgui, A. (1996). Role of NO in flow-induced remodeling of the rabbit common carotid artery. *Arterioscler.Thromb.Vasc.Biol.* **16**, 1256-1262.

Tseng, H., Peterson, T. E., & Berk, B. C. (1995). Fluid shear stress stimulates mitogen-activated protein kinase in endothelial cells. *Circ.Res.* **77**, 869-878.

Tzima, E., del Pozo, M. A., Kiosses, W. B., Mohamed, S. A., Li, S., Chien, S., & Schwartz, M. A. (2002). Activation of Rac1 by shear stress in endothelial cells mediates both cytoskeletal reorganization and effects on gene expression. *EMBO J.* **21**, 6791-6800.

Tzima, E., del Pozo, M. A., Shattil, S. J., Chien, S., & Schwartz, M. A. (2001). Activation of integrins in endothelial cells by fluid shear stress mediates Rho-dependent cytoskeletal alignment. *EMBO J.* **20**, 4639-4647.

Tzima, E., Kiosses, W. B., del Pozo, M. A., & Schwartz, M. A. (2003). Localized cdc42 activation, detected using a novel assay, mediates microtubule organizing center positioning in endothelial cells in response to fluid shear stress. *J.Biol.Chem.* **278**, 31020-31023.

Unger, E. F., Banai, S., Shou, M., Lazarous, D. F., Jaklitsch, M. T., Scheinowitz, M., Correa, R., Klingbeil, C., & Epstein, S. E. (1994). Basic fibroblast growth factor enhances myocardial collateral flow in a canine model. *Am.J.Physiol* **266**, H1588-H1595.

Valverius, E. M., Ciardiello, F., Heldin, N. E., Blondel, B., Merlo, G., Smith, G., Stampfer, M. R., Lippman, M. E., Dickson, R. B., & Salomon, D. S. (1990). Stromal influences on transformation of human mammary epithelial cells overexpressing c-myc and SV40T. *J.Cell Physiol* **145**, 207-216.

Veikkola, T. & Alitalo, K. (1999). VEGFs, receptors and angiogenesis. *Semin.Cancer Biol.* **9**, 211-220.

Versteeg, H. H., Nijhuis, E., van den Brink, G. R., Evertzen, M., Pynaert, G. N., van Deventer, S. J., Coffer, P. J., & Peppelenbosch, M. P. (2000). A new phosphospecific cell-based ELISA for p42/p44 mitogen-activated protein kinase (MAPK), p38 MAPK, protein kinase B and cAMP-response-element-binding protein. *Biochem.J.* **350 Pt 3**, 717-722.

Werner, G. S., Jandt, E., Krack, A., Schwarz, G., Mutschke, O., Kuethe, F., Ferrari, M., & Figulla, H. R. (2004). Growth factors in the collateral circulation of chronic total coronary occlusions: relation to duration of occlusion and collateral function. *Circulation* **110**, 1940-1945.

Weyts, F. A., Li, Y. S., van Leeuwen, J., Weinans, H., & Chien, S. (2002). ERK activation and alpha v beta 3 integrin signaling through Shc recruitment in response to mechanical stimulation in human osteoblasts. *J.Cell Biochem.* **87**, 85-92.

Whitelock, J. M., Murdoch, A. D., Iozzo, R. V., & Underwood, P. A. (1996). The degradation of human endothelial cell-derived perlecan and release of bound basic fibroblast growth factor by stromelysin, collagenase, plasmin, and heparanases. *J.Biol.Chem.* **271**, 10079-10086.

Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., & Ornitz, D. M. (1991). Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* **64**, 841-848.

Yu, Z. X., Biro, S., Fu, Y. M., Sanchez, J., Smale, G., Sasse, J., Ferrans, V. J., & Casscells, W. (1993). Localization of basic fibroblast growth factor in bovine endothelial cells: immunohistochemical and biochemical studies. *Exp.Cell Res.* **204**, 247-259.

Zhou, M., Sutliff, R. L., Paul, R. J., Lorenz, J. N., Hoying, J. B., Haudenschild, C. C., Yin, M., Coffin, J. D., Kong, L., Kranias, E. G., Luo, W., Boivin, G. P., Duffy, J. J., Pawlowski, S. A., & Doetschman, T. (1998). Fibroblast growth factor 2 control of vascular tone. *Nat.Med.* **4**, 201-207.

Acknowledgement

I would like to express my gratitude to Prof. Ulrich Pohl both for making this very interesting work possible and for stimulating discussions during the course.

A person I would like to thank very much is Dr. Torsten Gloe for inspiring this work and for his input of many good ideas. I want to thank him for always being willing to help and to shar his enormous amount of knowledge. Dr. Torsten Gloe has been an immense support during the lab research and has provided me with continuos motivation.

I want to thank Prof. Dr. Angelika M. Vollmar for her time and effort to be the superviser of this work.

I want to thank the GRK438, leaded by Prof. Wolfgang Siess, for providing me with financial support during the whole reseach period.

Furthermore, I would like to thank the following people:
Hanna Bridell and Nicole Hellwig for accompanying me through good times and bad times, for laughing and joking with me and for cheering me up. Especially thanks to Hanna Bridell for helpful discussions concerning the lab work. A thanks to the technical staff of our research group, Dorothea Gössel, Kasia Stefanofski, Brigitta Bscheider and Sabine D'arvis for helping in several situations. Moreover, I would like to thank Dr. Stefan Zahler, for introducing me to confocal microscopy and helping me with the FACS measurements. Also thanks to Dr. Petra Kameritsch for helping me with techinal questions regarding the confocal microscopy. Of course I would like to thank all the members of the research group of Prof. Pohl for creating an enjoyable and relaxing working atmosphere. Finally, I would like to thank my son Victor for letting my finishing this thesis and for not having problems with sleeping! And of course a special thanks to my husband Henrik for always supporting and helping me in any way.

Lebenslauf

Personal data

Name:	Christina Klarskov Mogensen
Maid name:	Andersen
Born:	09/11 1975
Place of birth:	Silkeborg, Denmark
Nationality:	Danish
Family status:	Marrid

Educational data:

Oct. 2002 – Dec. 2005:	Ph.D. Dissertation in the research group of Prof. Dr. U. Pohl, Institute of Physiology, LMU, Munich, Germany
Oct. 2004 – April 2005:	Maternity leave: Victor Mogensen (11/10 2004)
8. Nov. 2002:	Master of Science from the University of Aarhus, Denmark
Marts 2001 – Nov. 2002:	Master student at the August Krogh Institute, University of Copenhagen, Denmark
Sep. 2000 – Feb. 2001:	Biology student at the August Krogh Institute, University of Copenhagen, Denmark
Sep. 1998 – June 2000:	Biology student at the University of Aarhus, Denmark
July 1997 – June 1998:	Travel in Africa
Sep. 1995 – June 1997:	Biology student at the University of Aarhus, Denmark
Nov. 1994 – Apr. 1995:	Work + travel in Australia
Aug. 1991 – June 1994:	Senior High school, Silkeborg, Denmark
Aug. 1981 – June 1994:	Elementary school, Silkeborg, Denmark

Relevant job experience:

Sep. – Oct. 2001:	Instructor at a laboratory course in physiology for biology students at the University of Copenhagen, Denmark
Sep. – Oct. 2000:	Instructor at a laboratory course in physiology for biology students at the University of Copenhagen, Denmark
June – Aug. 2000:	Summer work at the department of the environment at the county of Aarhus, Denmark