Molecular mechanisms of thrombin-induced early and late-phase of ERK1/2 phosphorylation in vascular smooth muscle cells

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Introduction

Atherosclerosis is the underlying cause of most cardiovascular diseases, including coronary artery disease, ischemic gangrene and many cases of heart failure and stroke. These diseases are the principal cause of death in the developed countries [1]. Several genetic factors as well as acquired risk factors like smoking, hypercholesterolemia, diabetes mellitus and hypertension cause or accelerate the development of cardiovascular diseases. Atherosclerosis is an inflammatory disease and its lesions represent a series of highly specific cellular and molecular responses.

1.1 Pathology of atherosclerosis

Atherosclerosis, a pathologic and progressive process affecting the arterial walls, is characterized by the accumulation of lipid particles and immune cells in subendothelial regions, and by the proliferation of vascular smooth muscle (VSM) cells, leading to a narrowing of the arterial lumen, following plaque rupture and thrombosis [2].

The early phase of atherosclerosis is an inflammatory response induced by a retention of lipoproteins in the arterial intima. An endothelial dysfunction, which is the first step in the disease, can be induced by a variety of risk factors such as elevated levels of modified

low density lipoprotein (LDL), free radicals caused by smoking, hypertension, diabetes, genetic functions, infectious microorganisms, hemodynamic factors, sex hormones, or a combination of these and other factors [3, 4]. This dysfunction alters the adhesiveness and permeability of the endothelium. Lipoproteins invade into the arterial wall and are retained in the extracellular matrix [5]. There oxidated LDL activates endothelial cells [6], which express several types of leukocyte adhesion molecules, and thereby causes the adhesion of blood cells at the site of activation [7]. Once adherent to the endothelium, leukocytes migrate into the intima in response to chemoattractant stimuli [8, 9]. The macrophage–colony stimulation growth factor produced in the inflamed intima, induces a differentiation of monocytes into macrophages [10], which produce reactive oxygen species [11] and secrete proteolytic enzymes that may degrade matrix components. The loss of matrix components may lead to destabilization of plaques and, thus, to a risk for plaque rupture and thrombosis [12, 13]. Under these conditions, macrophages engulf the modified lipoproteins and become foam cells. Moreover, the VSM cells are attracted into the lesion by chemotactic factors generated by the activated macrophages and endothelial cells, such as platelet–derived growth factor (PDGF), fibroblast growth factor 2 (FGF2) and transforming growth factor β (TGFβ). Following this intrusion, VSM cells change their phenotype, proliferate and secrete fibrous elements and extracellular matrix components. This initial lesion represents the primary state of the disease and is designated "fatty streak" [14] (Fig. 1.1). The further progression of the "fatty streak" to intermediate and advanced lesions is characterized by the formation of a fibrous cap that protects the lesion from the lumen. The fibrous cap coats the necrotic core, which is composed of a mixture of leukocytes, lipids, debris and VSM cells. The lesions expand by means of continued leukocyte adhesion and proliferation of VSM cells. The relative proportion of VSM cells, macrophages and lipid in the necrotic core, and the thickness of the fibrous cap determine the stability of the plaque. A high proportion of inflammatory cells and lipids, and lower proportion of VSM cells result in an unstable plaque, which can easily be broken, leading to thromboembolic complications [15].

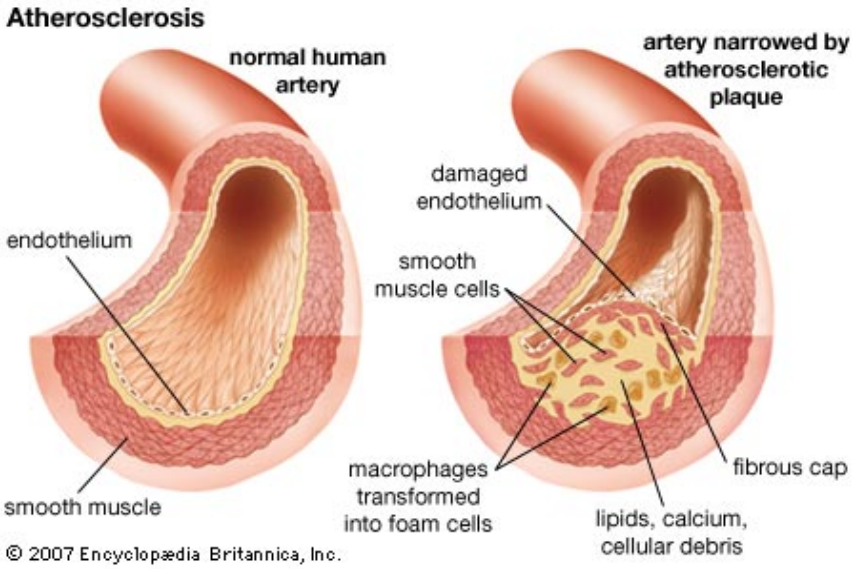

Figure 1.1: Damage in the artery by atherosclerosis. Healthy artery and atherosclerotic artery are presented in the cross-sectional picture of human blood vessels.

Thinning of the fibrous cap is influenced by VSM cell apoptosis and the continuous influx and activation of macrophages, which release metalloproteinases and other proteolytic enzymes [16]. In contrast, thick fibrous caps stabilize the plaques. In this situation, VSM cells can change their phenotype from a synthetic back to a contractile one leading to additional plaque stabilization [4] (Fig. 1.1).

1.2 Smooth muscle cells

1.2.1 Smooth muscle cell structure

Smooth muscle (SM) cells are spindle–shaped, and can contract and relax. SM cells are of 50–400 μm length and 2–10 μm width. They possess only one nucleus, and are, unlike the skeletal muscle cells, arranged in sheets or bundles where single cells are connected by gap junctions. The cells contain actin filaments and myosin, which are organized by the intermediate filament proteins vimentin and desmin. Specific isoforms of both contractile proteins are expressed in SM cells. The sarcolemma contains caveolae, which are often in

close proximity to the sarcoplasmic reticulum or to mitochondria and have been proposed to organize signaling molecules in the membrane [17].

1.2.2 Vascular smooth muscle cell phenotype

VSM cells are highly specialized contractile cells that regulate blood vessel tone–diameter, blood pressure, and blood flow in response to physical, hormonal, neurotransmitter–relayed or paracrine stimuli. In adult blood vessels, VSM cells exhibit a low rate of proliferation and low synthetic activity. They express contractile proteins, ion channels and signal molecules required for the contractile function of the cell. However, the cells are not terminally differentiated and hold plasticity, and, thus, can undergo rather profound and reversible changes in phenotype in response to local changes [18].

The VSM cell plasticity is important during vascular development because the cells play a key role in the morphogenesis of blood vessels. Under these conditions, the cells are dedifferentiated and exhibit a high rate of proliferation, migration and production of extracellular matrix components such as collagen, elastin and proteoglycans [19]. However, in mature blood vessels, VSM cells exhibit a contractile or differentiated phenotype which is characterized by the expression of contractile markers such smooth muscle (SM) α–actin, smooth muscle myosin heavy chain (SM–MHC), h–caldesmon and calponin.

Upon arterial injury, differentiated VSM cells can undergo changes from a contractile to a synthetic phenotype and increase their rate of cell proliferation, migration, and synthetic capacity playing a critical role in vascular repair [18]. However, the high degree of plasticity in VSM cells predispose the cells to abnormal environmental signals that can lead to an anomalous phenotype and acquisition of characteristics that can contribute to development of vascular diseases [20]. During atherosclerosis, the exposure to specific growth factors and inflammatory mediators induce the dedifferentiation of VSM cells. Under these conditions, dedifferentiated VSM cells migrate into the intima, start to proliferate, produce

extracellular matrix components and contribute to the formation of the fibrous cap over the atherosclerotic plaque [16].

1.2.3 Phenotypic modulation of vascular smooth muscle cells

The phenotypic modulation of VSM cells is regulated by a complex interaction of multiple factors like cell–cell interactions, extracellular matrix (ECM) components, humoral factors, local chemical conditions, and mechanical forces. However, phenotypic modulation of the cells during atherosclerosis also involves other factors including growth factors and cytokines, inflammatory cell mediators, lipids, lipid peroxidation products, or reactive oxygen species.

In the media, VSM cells are differentiated and encased by a complex, highly structured ECM composed of elastin, type I and III collagen, and proteoglycans [21]. The VSM cells bind to the ECM via specific integrin receptors and this binding is crucial for maintaining the cells in a differentiated phenotype [22] and to modulate the cell function. Biochemical signals such as growth factors, must pass through the ECM to be released or sequestered. Additionally, laminin, collagen type I and heparan sulphate species inhibit VSM cell proliferation [23, 24, 25, 26]. However, glycoproteins associated with atherosclerotic lesions (osteopontin, SPARC, thrombospondin, tenascin, collagen type VIII) induce migration and changes in the phenotype of VSM cells [27, 28, 29, 24, 30, 31]. Apart from the ECM components, other factors have important effects on the phenotypic modulation of the cells. Cytokines and growth factors secreted by macrophages (Interleukin–1 (IL–1), tumor necrosis factor (TNF) α, TGFβ and T cells are important regulators of VSM cell migration, proliferation and extracellular matrix production. Application of mechanical forces can actively change the phenotype of the cells [32]. Under elevated blood pressure, the mechanical stress is increased on the vascular wall, and mechanical strain is a mitogenic stimulus for VSM cells [33].

The peptidic growth factors and cytokines PDGF [2], basic fibroblast growth factor (bFGF) [34], heparin–binding epidermal growth factor like growth factor (HB–EGF) [35], insulin–like growth factor–1 (IGF–1) [36], IL–1 [37], TNFα [38] and TGFβ [39] can induce VSM cell proliferation and are absent or only weakly expressed in the normal artery, whereas they are upregulated in atherosclerotic lesions. In addition, PDGF, IGF–1 and accumulation of reactive oxidative species (ROS) can induce VSM cell chemotaxis [40]. The

Table 1.1: Factors involved in VSM cells phenotype modulation.

Differentiation	Proliferation	
Collagen type I and III	bFGF	Osteopontin
ECM	Collagen type VIII	PDGF
Heparan sulphate species	HB-EGF	SPARC
Laminin	IGF-1	T cells
Serum	IL-1	Tenascin
Thrombin	Mechanical forces	TGFβ
	Thrombospondin	TNFα
	ROS	

intracellular mechanisms of the phenotype regulation in VSM cells are complex and only poorly understood. The PDGF–BB–induced VSM cell dedifferentation has been shown to be mediated by multiple mechanisms, including expression of Krüppel–like zinc finger factor type 4 (KLF4) [41, 42], specific protein 1 (Sp1) [43], HES–related repressor protein (HERP1) [44], and phosphorylation of Elk–1 by extracellular signal–regulated protein kinases (ERK)1/2 activation [45]. Oxidized phospholipids are concentrated within atherosclerotic lesions and induce the suppression of VSM marker genes by nuclear translocation of KLF4 [46].

Similarly, the signalling cascade involved in VSM cell differentiation is still intensively investigated. Initially it was shown that the activation of protein kinase G (PKG) [47, 48] or of the insulin–like growth factor receptor (IGFR) by insulin and subsequent activation of the phosphoinositide 3–kinase pathway (PI 3–kinase)/AKT, also called protein kinase B (PKB) [49] could be involved in promoting differentation of the cells. Endothelial cell co-

culture promotes a differentiation of VSM cells, possibly via activation of the AKT pathway [50]. However, these results contradict with Reusch et al. who demonstrated that serum and thrombin can promote a redifferentiation in VSM cells and require G protein–coupled receptor– (GPCR) mediated activation of the Ras/Raf/MEK/ERK signaling pathway [51].

Interestingly, the VSM cell phenotype can be modulated by the activation of mitogen–activated protein kinases (MAPK) by multiple and different stimuli [45, 51, 52, 53], attributing this signaling pathway an important role in the regulation of the VSM cells phenotype.

1.3 The ERK/mitogen–activated protein kinase signaling pathway

The canonical Ras–Raf–MEK–ERK/MAPK signaling pathway is an evolutionarily conserved pathway that is involved in the control of many fundamental cellular processes including cell proliferation, survival, differentiation, apoptosis, motility and metabolism [54]. Activation of the ERK cascade is central to control of G0–G1 cell–cycle transition and the passage of cells trough mitosis or meiosis. The MAPK pathway transmits signals from cell surface receptors to ERK/MAPK, which distribute them to different effectors.

Multiple cell–surface receptors activate Ras GTPases. Ras GTPases comprise a family of membrane–resident proteins that shuttle between an inactive GDP–binding and active GTP–binding conformation [55] and are encoded by potent proto–oncogenes. Ras is primarily activated by the guanine nucleotide exchange factor Sos. The adaptor protein growth factor receptor–bound protein 2 (Grb2) forms a complex with Sos by its Src homology (SH) 3 domains. The Grb2/Sos complex is recruited by activated receptor tyrosine kinase (RTK) through binding of the Grb2 SH2 domain to specific pTyr sites of the receptor. This translocation of Grb2/Sos complex to the plasma membrane brings Sos closer

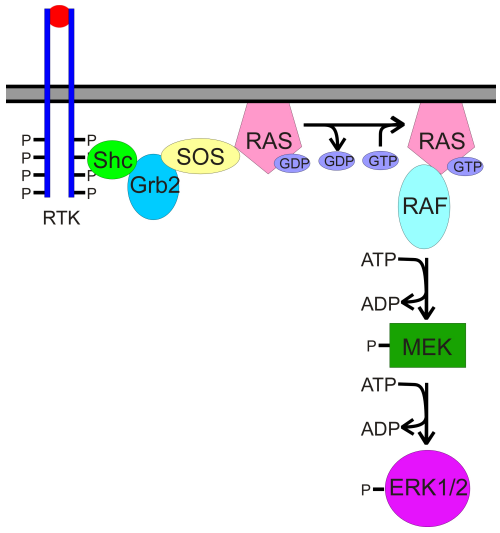

Figure 1.2: The ERK/MAPK signaling pathway. Upon RTK stimulation, the Grb2/Sos complex is recruited by Src. Nearing of the plasma membrane, Grb2/Sos complex induces Ras activation and, subsequently, ERK1/2 phosphorylation.

to Ras and promotes the exchange of GTP for GDP [56, 57] (Fig. 1.2). The recruitment of Grb2/Sos complex can be additionally triggered by binding of the complex to Shc. Shc is another adaptor protein that forms a complex with many activated receptors through its phosphotyrosine–binding (PTB) domain [58]. Alternatively, membrane–linked docking proteins such as insulin receptor substrate–1 (IRS1) or fibroblast growth factor receptor substrate (FRS) 2α can be phosphorylated in response to activation of certain RTKs and recruit the Grb2/Sos complex to the plasma membrane [59, 60]. GTP–bound Ras can interact with different proteins including the serine/threonine kinase Raf or phosphatidylinositol 3–kinase (PI3K) to stimulate numerous intracellular processes [61] (Fig. 1.2). All three Raf family members can activate the dual specificity mitogen–activated protein kinase kinase 1/2 (MEK1/2) by phosphorylating them at two serines in the MEK activation loop. MEK is bound to ERK1/2 in its inactive state. Upon activation, MEK activates ERK/MAPK by phosphorylation of Thr183 and Tyr185 residues at the activation loop, which leads to dissociation of ERK1/2 from MEK [62]. Activated ERK/MAPK phosphorylates a variety of cytoplasmic and membrane linked substrates (e.g. EGFR, Sos).

Additionally, ERK1/2 are rapidly translocated into the nucleus where they phosphorylate and activate transcription factors [63]. Nuclear localization of ERK1/2 appears to be obligatory for correct cellular responses [64]. ERK1/2 is deactivated through the action of serine/threonine, tyrosine [65] or dual specificity phosphatases [59, 66]. The relocalization of inactive ERK1/2 to cytoplasm, which is necessary for the next stimulation, involves a MEK–dependent active transport. MEK exhibits a nuclear export signal (NES) and can shuttle between the cytoplasm and the nucleus. It transiently enters the nucleus and bind inactive ERK1/2 to export it to the cytoplasm [67]. One possible way of ERK1/2 activation involves GPCR stimulation.

1.4 Activation of MAPK by G protein–coupled receptors

The activation of the MAPK signaling pathway by GPCRs plays an important role in multiple cellular processes. GPCRs comprise the largest group of cell–surface receptors [68] and share a common transmembrane topology with seven transmembrane-spanning helices with an extracellular amino terminus and an intracellular carboxy terminus. Therefore, these receptors are also referred to as heptahelical receptors or 7TMRs. Many hormones, neurotransmitters or other intercellular messengers, including hormonal peptides and glycoproteins, chemokines, bioactive lipids, eicosanoids, proteases, amino acids, nucleotides, biogenic amines, and ions can bind specifically to GPCRs and thereby constitute a rich network of information exchange throughout the organism [69, 70, 71, 72, 73]. Depending on the agonist and target tissues, GPCRs play a crucial role in the regulation of multiple fundamental physiological functions such as exocrine and endocrine secretion, smooth muscle and cardiac contraction, neuronal excitability and others [71, 74].

The external signals are transduced from GPCRs to intracellular effectors by the het-

erotrimeric guanine nucleotide–binding regulatory proteins (G proteins), composed of α, β and γ subunits. According to their α subunits G proteins are classified into four subfamilies: G_s, $G_{i/o}$, $G_{q/11}$, and G_{12}. The binding of an agonist to GPCRs induces a movement of the transmembrane–spanning segments and promotes a conformational change in the cytoplasmic domain of the receptor leading to the exchange of GDP to GTP at the α subunit of the G protein [75, 76] (Fig. 1.3).

Adenylyl cyclases, phospholipase C (PLC) isoforms, and ion channels can be stimulated by the resulting GTP–G_α andor G$\beta\gamma$ subunits which transduce GPCR activation into biological responses, including mitogenesis. Inactivation of the receptor depends on the GPCR kinases (GRKs) and arrestins, which initiate GPCR phosphorylation, internalization and intracellular sorting towards endolysosomal degradation or endosomal recycling [77, 78], as well as of the rate of GTP hydrolysis by the G_α subunit (Fig. 1.3).

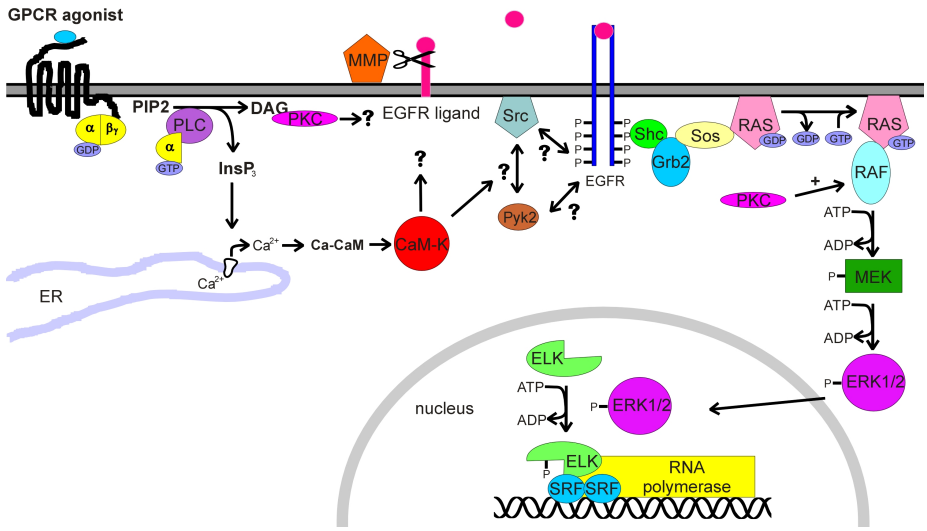

Figure 1.3: GPCR–induced EGFR transactivation and ERK1/2 phosphorylation. Agonist binding to GPCR activates the signaling cascade. After stimulation, GPCR activates diferent G proteins, which induces PLCβ activation. $InsP_3$ and DAG, produced by activated PLCβ induce Ca^{2+} mobilization and activation of the PKC and calcium/calmodulin–dependent kinase (Ca/CaM). The details of the agonist–induced shedding of EGFR ligands and EGFR transactivation remain unknown. EGFR dimerization and autophosphorylation induces ERK1/2 phosphorylation by activation of the MAPK signaling pathway. Activated ERK1/2 is translocated into the nucleus, where it phosphorylates several transcription factors such Elk–1 and promotes the transcription of specific target genes.

Members of the phospholipase C (PLC) family (β, γ, δ, and ϵ) can be activated after the stimulation of many GPCRs. For instance, the GTP bound–$G_{\alpha\ q/11}$ activates the β isoforms of PLC by interaction with the carboxy–terminal region of the enzyme [79]. PLCβ can be also stimulated by G$\beta\gamma$ and G_i. Activated PLC catalyzes the hydrolysis of phosphatidylinositol 4,5–bisphosphate (PIP2) to produce two second messengers: inositol 1,4,5–trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG). Ins(1,4,5)P_3 binds to intracellular receptors at the endoplasmic reticulum (ER) and induces a mobilization of Ca^{2+} from ER stores leading to a rapid increase in the intracellular concentration of Ca^{2+} followed by a slower Ca^{2+} entry from outside [80]. DAG, the other second messenger generated by PLC, activates diverse isoforms of the PKC, a phospholipid–dependent protein Ser/Thr–kinase family differentially expressed in cells and tissues. A region in the regulatory domain of PKC binds to PIP2 contributing to the membrane translocation of the enzymes [81].

Multiple studies tried to explain the activation of the ERK/MAPK signaling pathway by GPCR stimulation showing that GPCRs can induce ERK/MAPK activation via PKC–dependent and PKC–independent pathways [69, 82, 83]. Although it is well accepted that PKC induces the Ras/Raf/MEK/ERK cascade, the details how PKC–mediated signals are propagated remain incompletely understood. Many studies reported that PKC activates the cascade through a Ras–dependent pathway [84, 85, 86] whereas other reports suggested that PKC–mediated RKIP (raf kinase inhibitory protein) phosphorylation induces the dissociation of the inhibitor from Raf leading to Raf activation and subsequently MEK/ERK phosphorylation [87, 88].

In addition, PKC can modulate and inhibit the activity of the epidermal growth factor receptor (EGFR) by phosphorylation of Thr–654 at the juxtamembrane region of the receptor [89, 90, 91]. In this manner, the intensity and/or duration of the GPCR–induced EGFR transactivation can be modulated by PKCα.

The ERK/MAPK signaling pathway can be activated in a PKC–independent fashion

by receptor tyrosine kinases (RTK) transactivation [92]. In addition, non–RTK are involved in the transduction of the signal, including Src, focal adhesion kinases (FAK), and proline–rich tyrosine kinase 2 (Pyk2), which promote ERK activation via tyrosine phosphorylation of adaptor proteins. The EGFR has been identified as a necessary signal transduction element of GPCR–mediated mitogenic signaling [93]. In VSM cells, EGFR phosphorylation is induced by angiotensin II and thrombin stimulation, which was first reported in primary rat SM cells [94]. The EGFR family has four members: EGFR or ErbB1/HER1, ErbB2/Neu/HER2, ErbB3/HER3 and ErbB4/HER4. They exhibit an extracellular ligand–binding domain, a single membrane–spanning region and a cytoplasmatic protein tyrosine kinase domain. The EGF–related peptide growth factors from the family of EGFR ligands, including epidermal growth factor (EGF), HB–EGF, amphiregulin, epiregulin and TGF–α, bind to the extracellular domain of the EGFR leading to the formation of receptor homo– and heterodimers [95]. After EGFR activation and dimerization, the intrinsic kinase activity of the receptor results in an autophosphorylation of various tyrosine residues at positions 992, 1068, 1086, 1148 and 1173 [96]. These phosphorylated tyrosines are docking sites for SH2 domain–containing proteins like p120–GAP, Shc, Grb2 or Src [97].

In many cases, the transactivation of the EGFR by GPCR stimulation is mediated by shedding of precursor forms of the EGFR ligands HB–EGF, TGF–α or amphiregulin [98, 99, 100] from the plasma membrane. All these growth factors are synthesized as transmembrane precursors that are cleaved proteolytically to yield the respective soluble and active growth factors. The shedding of the growth factor precursors is induced by activation of members of the zinc–dependent a disintegrin and metalloproteinase (ADAM) family such as ADAM 9, ADAM 10 [101], ADAM 12 [102], ADAM 17 [103, 104], or by activation of the matrix metalloproteases (MMP) MMP–2, MMP–3 and MMP–7 [105]. The precise mechanisms by which GPCR induce the ADAM activation remains elusive. Classical G protein effectors, such as PKC, could be involved in MMP activation [106,

107], whereas PI3K and Src–family non–RTK could be early intermediates in the EGFR transactivation [68, 108, 109].

1.5 Heparin–binding EGF–like growth factor

HB–EGF is a member of the EGF superfamily of peptide growth and differentiation factors [110] and was first identified as a secreted product of macrophage–like U–937 cells [111]. Pro–HB–EGF is a 20–22 kDa glycoprotein and binds to the EGFR, thereby inducing its autophosphorylation.

HB–EGF is primarily expressed as a 208 amino acid (AA) translation product (pre–pro–HB–EGF) [111] and, like other members of the EGF family, is synthesized as a transmembrane protein that can be cleaved at the plasma membrane to yield soluble HB–EGF (sHB–EGF) [112]. Analysis of the pre–pro–HB–EGF structure revealed that HB–EGF is subdivided into different domains including the signal peptide (AA 1–23), the propeptide (AA 24–62), the sHB–EGF domain (AA 63–149), a juxtamembrane (AA 150–160), a transmembrane (AA 161–184) and a cytoplasmatic (AA 185–208) domain [113]. sHB–EGF possesses an amino acid motif of about 40–47 AA containing six cysteine residues known as the EGF–like domain [114], which mediates HB–EGF binding to EGFR and subsequent growth factor activition. Singular characteristics of HB–EGF are its ability to bind heparin and cell surface heparan–sulfate proteoglycans (HSPGs) [111, 115] (Fig. 1.4). The ectodomain shedding of HB–EGF is regulated by several stimuli like thrombin, calcium ionophores [116], various growth factors and cytokines [113] and intracellular signalling pathways like the MAP kinase signaling cascade [107, 117, 118, 119]. Pro–HB–EGF is processed by MMP–2, 3, 7 and ADAM 9, 10, 12, 17 [101, 102, 103, 107, 120, 121, 122, 123]

Although HB–EGF was first identified as a soluble mitogenic growth factor, pro–HB–EGF exhibits different functions as specific receptor for diphtheria toxin [124, 125], and juxtacrine growth factor that interacts with neighbouring cells in a non–diffusible man-

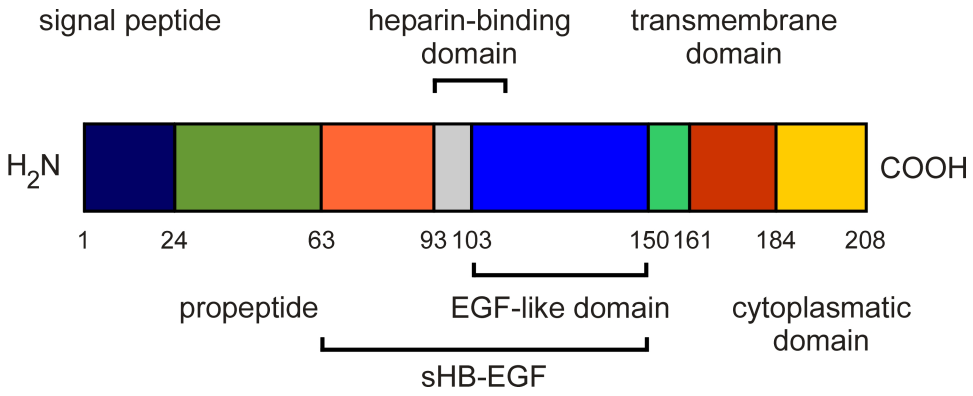

Figure 1.4: Structure of pre–pro–HB–EGF. The domain structure of the primary translation product of pre–pro–HB–EGF is depicted. Pre–pro–HB–EGF consists of a signal prepeptide, a propeptide, mature HB–EGF, and juxtamembrane, transmembrane and cytoplasmatic domains.

ner [126, 127]. The juxtacrine activities of transmembrane HB–EGF are modulated by CD9, a member of tetraspanning protein family [128, 129]. The fact that the complex between CD9, pro–HB–EGF and the EGFR are localized at cell–cell contact sites indicates that pro–HB–EGF interacts in intracellular communication in a juxtacrine manner [126]. Additionally, some studies revealed that the cytoplasmatic domain of pro–HB–EGF can play a role in HB–EGF function. BAG–1, a multifunctional protein, can bind to the cytoplasmatic domain of pro–HB–EGF and increase HB–EGF secretion [130]. Other studies demonstrated, that the cytoplasmic domain of HB–EGF is phosphorylated by external stimuli, and that these phosphorylation sites are involved in HB–EGF–dependent tumorigenesis [131].

sHB–EGF is a potent stimulator of cell proliferation and migration and is expressed in a variety of tissues and in a large number of cells like vascular endothelial cells, SM cells, epithelial cells, skeletal muscle, inflammatory cells, renal mesangial cells, keratinocytes and tumor cells [113, 115]. Interestingly, HB–EGF is a critical factor in the transactivation of EGFR by many GPCR ligands (endothelin–1, angiotensin–II or thrombin) resulting in the Raf/MEK/ERK canonical signaling pathway activation [98, 132]. Additionally, HB–EGF might stimulate cells by inducing the transcription of other growth factors and growth

factor receptor genes. In SM cells, for example, HB–EGF induces an upregulation of SMC mitogens like PDGF and FGF–2 [133]. In addition, the C–terminal fragment of HB–EGF generated after HB–EGF shedding, can be translocated to the nucleus and promote S–phase entry by sequestering promyelocytic leukaemia zinc finger protein, a transcriptional repressor of cyclin A [134, 135].

The expression of HB–EGF is highly regulated and a number of regulatory factors have been described, some of which suggest roles of HB–EGF in physiological and pathological processes [113], including atherosclerosis [136]. The fact that HB–EGF expression is increased in atherosclerotic plaques [136], suggest that the growth factor may be involved in the development of the disease.

1.6 Tyrosine kinases

Protein tyrosine kinases (PTK) represent a diverse and rapidly expanding superfamily of proteins, including both RTK, such as EGFR, and soluble cytoplasmic enzymes also known as non–RTK, such as FAK, Src, and Pyk2.

PTK are enzymes which catalyze the transfer of the gamma–phosphate group of ATP to the hydroxyl groups of specific tyrosine residues in peptides and control many different cellular functions like gene expression, metabolic pathways, cell growth and differentiation, membrane transport and apoptosis. The kinase activity can be modulated by phosphatases, upstream kinases, cofactors and ligands and by interactions with other activating or regulatory proteins [137].

1.6.1 Non–receptor tyrosine kinases

The non–RTK (currently known 32 proteins in nine subgroups) respond to extracellular stimuli by modular units like Src homology 2 (SH2), Src homology 3 (SH3) and Pleckstrin homology (PH) domains, or modification by lipids (e.g. myristate), for appropriate

subcellular localization [138].

Non–RTK are organized into multiple domains. The kinase domain has a length of approximately 300 residues and consists of an N–terminal lobe containing a five–stranded β sheet, an helix (αC), and a larger, mostly helical C–terminal lobe. This domain is highly conserved among receptor and non–RTK, and is similar to those of serine/threonine and dual–specificity protein kinases [139]. The second most common functional domain is the SH2 domain. This region comprises approximately 100 AA and forms structures capable of high–affinity binding to phosphotyrosine–containing peptide sequences that promote protein modification–dependent protein–protein interactions [140]. Proline–rich peptide sequences are docking sites for the SH3 domain which has a length of 50 AA, and bind approximately nine residues in length of the docking site, that possess a left–handed polyproline type II helix structure [141].

Src

Src was the first cellular protein tyrosine kinase identified and has served as a prototype of the Src family kinases (SFKs) [142]. The Src family contains eight members and is separated in two subfamilies according to their sequences and tissue expression. Src, Yes, Fgr and Fyn, are ubiquitously expressed, whereas Lyn, Lck, Hck and Blk are mainly expressed in non–adherent cells of the haematopoietic system [143, 144, 145]. SFKs play key roles in the regulation of cell proliferation and survival, as well as in cell adhesion, cell morphology and motility [146, 147]. The Src family structure is characterized by four highly conserved SH domains termed SH1 to SH4. The N–terminal sequence designated as SH4 domain contains fatty acid modification sites. The proteins can be modified by myristoylation (irreversible) or palmitoylation (reversible).This post–translational modification determines the membrane localization of Src. The neighboring domain is a region in which the sequences of Src family proteins show the lowest similarity. This region determines the unique functions of the different family members by regulating their activity and is,

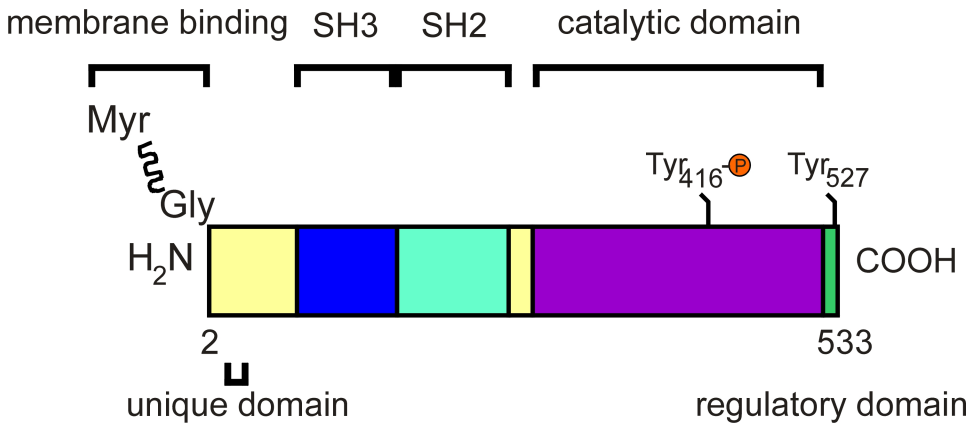

Figure 1.5: Structural features of Src. The amino terminus of the protein is myristoylated at Gly2. Positions of the membrane binding domain, the unique domain, the SH2, the SH3, the catalytic and regulatory domains are indicated.

thus, referred to the unique domain [148]. Next to the N–terminal region an SH3 domain is located followed by an SH2 domain, which is followed, in turn, by a tyrosine kinase domain (SH1). The C–terminal part of the Src family contains a tyrosine residue (Tyr527) that regulates the activity of the protein and is termed regulatory domain [149, 150] (Fig. 1.5).

The kinase activity of Src is regulated by tyrosine phosphorylation. Tyr527 is phosphorylated by COOH–terminal Src kinase (Csk), another non–RTK. This phosphorylation causes an intramolecular interaction with the SH2 domain of Src [151, 152]. In this conformation, both the SH2 and the SH3 domain are engaged intramolecularly, and the surface of the catalytic domain of the kinase is not accessible for interaction with other proteins. Competitors for the SH2 or SH3 domains cause an opening of c–Src and partial activation of its kinase activity as does dephosphorylation of Tyr527 by protein tyrosine phosphatases (PTP) [146, 153, 154]. Complete activation of Src requires, in addition, autophosphorylation at Tyr416, which is located in the activation loop of the catalytic center. Phosphorylation of Tyr527 by the C–terminal Src kinase, CSK, together with dephosphorylation of Tyr416 by PTP, negatively regulates the c–Src kinase activity [155, 156, 157, 158].

Src is a versatile enzyme that is involved in signaling processe activated by many RTKs including the PDGF receptor, the EGFR, the fibroblast growt factor receptor (FGFR), the

stem cell factor receptor (SCFR) and others [159], and induces mitogenesis by initiation of signaling pathways required for DNA synthesis [160], control of receptor turnover [161], actin cytoskeleton rearrangement [162] and motility [163], and survival [164]. The interaction between c–Src and RTKs is complex and bidirectional. Interestingly, Src is not only activated by the EGFR, but can also modulate the activity and signaling of this receptor. After EGFR activation, dimerization and autophosphorylation, Src binds to the receptor via its SH2 domain. This EGFR–induced Src activation can lead to additional tyrosine phosphorylation of the EGFR at positions Tyr845 and Tyr1102 [165], which is required for EGFR signaling and mitogenesis [166, 167].

Src is not only activated by RTKs, but also GPCRs can activate Src by different mechanisms, like direct association between Src and the receptor. Moreover receptor–associated proteins can lead to the transactivation of the RTKs and focal adhesion complexes by G–protein–mediated signals. In addition, GPCR trafficking and effects on cell proliferation and cytoskeletal reorganization are controlled by Src activity [140].

Pyk2

Pyk2 and FAK define another non–RTK family. Whereas FAK is expressed ubiquitously, Pyk2 is primarily expressed in brain and, to a lower extent, in liver, lung, kidney, SM cells and hematopoietic cells [168, 169, 170]. Pyk2 (112 kDa) is slightly smaller than FAK. The sequence conservation in the central kinase domain and the surroundings of the conserved tyrosine phosphorylation sites of both proteins suggest that these phosphorylated tyrosines are binding sites for SH2–domain containing proteins. The N–terminal region of the FAK–family contains the FERM domain, which is capable of mediating interactions between these proteins and transmembrane receptors [171, 172]. The C–terminal regions of FAK and Pyk2 contain two proline–rich domains wich are binding sites for SH3 domain–containing proteins like p130Cas [173] or the GTPase–activating protein, Graf [174]. Additionally, the C terminal region contains a consensus paxillin binding sequences (PBS1, 875–894 and

PBS2, 985–999) [175, 176] (Fig. 1.6). Pyk2 is known to be a unique protein–tyrosine kinase activated upon an increase in the cytoplasmic free–Ca^{2+} concentrations after stimulation of cells with ligands such as lysophosphatidic acid, endothelin, vasopressin and PDGF that bind to receptors linked to PLC activation [170]. The ability of Pyk2 to respond to Ca^{2+} enables it to function as a link between heterotrimeric GPCRs and downstream signalling pathways like the MAP kinase signalling cascade [177, 178]. Additonally, activated Pyk2 is recognized by the SH2 binding site of Grb2. Grb2 association with Sos directly link the activation of the Ras/Raf/MEK/ERK canonical cascade with the Pyk2 activation [179, 180] (Fig. 1.3).

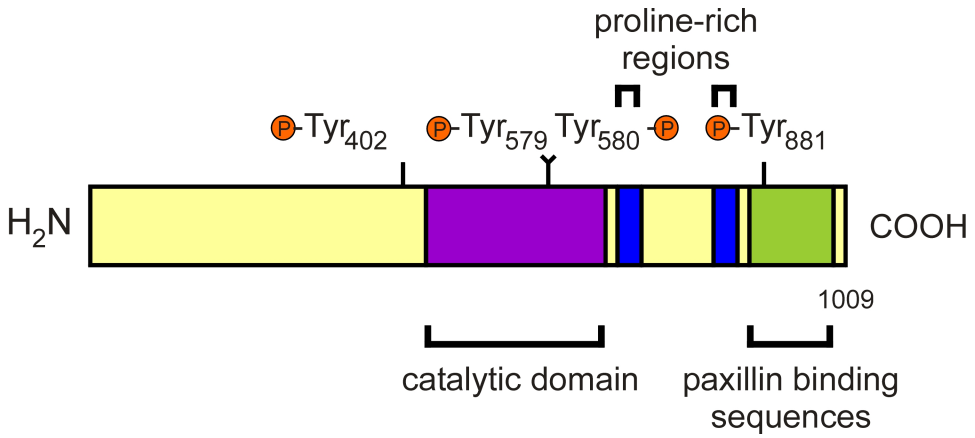

Figure 1.6: Schematic representation of Pyk2 sequence. Positions of the catalytic domain, proline–rich domains and paxillin binding sequences are indicated along with Tyr residues that can be phosphorylated.

1.7 MAPK signaling pathway and rediffernentiation

As described before, several studies demonstrated the involvement of RTKs and the Ras/-Raf/MEK/ERK cascade in signaling processes involved in cell proliferation and differentiation. Interestingly, the same receptors can induce proliferation or differentiation depending on their cellular expression pattern, whereas the ERK/MAPK–induced differentiation or proliferation depends on the agonist. In PC12 cells, fibroblast growth factor

or nerve growth factor (NGF) stimulation leads to abrogation of cell proliferation [181], whereas EGF treatment induces cell division [182]. Since both growth factors activate the ERK/MAPK pathway, an intensive analysis of the signal transmission was perform which revealed that NGF induces a sustained ERK activation, whereas the EGF–induced ERK activation is transient [183]. Factors that may be involved in controlling the duration of ERK activation include the concentration of ligands [184, 185] or/and receptors which could change the transient ERK activation into a sustained one. Additionally, the rate of receptor internalization can play a critical role in the duration of the ERK activation. The consequence of the sustained ERK activation is the translocation of the activated kinase into the nucleus [183, 185, 186, 184] where ERK phosphorylates and activates multiple transcription factors, many of them related to the redifferentiation of cells and induces a gene expressions which is not observed in response to transient activation.

As explained above, the progression of vascular diseases is typically accompanied by VSM cell proliferation and a phenotypic modulation, characterized by a loss of contractile function and a local matrix degradation [187].

It is known that the dedifferentiation of VSM cells is characterized by a low expression rate of contractile proteins such as SM–specific α–actin and myosin heavy chain (SM–MHC). Neonatal VSM cells exhibit a high expression profile of contractile proteins in the presence of serum and thrombin [51], whereas PDGF chiefly induces a proliferative response. A more detailed analysis revealed that thrombin stimulation induces a biphasic ERK activation (called before sustained activation). However, PDGF stimulation leads to Akt activation via PI3K [51]. Akt inactivates Raf by phosphorylation at Ser259 [188, 189] and, subsequently, the ERK activation is transient and the kinase is not translocated into the nucleus.

Chapter 2

Aims

Despite recent progression in the understanding of mechanisms that define the development of vascular diseases and changes induced in the VSM cell phenotype, the molecular mechanisms of the signal transduction remain incompletely described. A previous study demonstrated that serum– and thrombin–induced SM–α actin and SM–MHC expression depend on the long–lasting ERK1/2 activation which leads to the phosphorylation of several transcription factors and promotes gene transcription [51, 190]. Although it is known that a biphasic ERK1/2 activation can lead to cell differentiation [97], the molecular mechanisms of the cessation and reappearance of the ERK1/2 phosphorylation remained unknown.

Therefore, the main purpose of this study was to elucidate the details of the ERK1/2 activation by thrombin stimulation. The abolishment of the thrombin–induced second phase of ERK1/2 activation by treatment with transcription inhibitors demonstrated the critical role of *de novo* protein expression in the signalling pathway. An analysis of *de novo* protein expression of VSM cells by thrombin stimulation revealed an upregulation of the HB–EGF expression, which is coincident with the second phase of the ERK1/2 activation. The crucial role of the HB–EGF expression for the long lasting ERK1/2 phosphorylation was confirmed by HB–EGF siRNA treatment of the cells.

Although several studies have reported GPCR–mediated EGFR transactivation, the

details of this process remain elusive. In this study, it is demonstrated that Src is involved in the thrombin–induced ERK1/2 activation. However, EGFR transactivation by thrombin stimulation requires the "triple membrane–spanning" pathway. This places Pyk2 and Src downstream of the EGFR. Finally, the intermediate role of Pyk2 in the EGFR–induced Src activation was demonstrated by Pyk2 knockdown in VSM cells.