1 Introduction

1.1 G protein-coupled receptors

G protein-coupled receptors (GPCRs) form the largest protein family of receptors in the body. Nearly 2000 members of this superfamily have been identified in vertebrates (Bockaert and Pin, 1999; George et al., 2002). In man about 800 genes encode non-olfactory GPCRs, representing 3-4% of the human genome. The GPCRs are activated by a wide variety of endogenous ligands, including hormones, growth factors, peptides, nucleotides, neurotransmitters, phospholipids, fatty acids, ions and proteases. Also exogenous stimuli, such as photons, taste and odorant molecules mediate their actions *via* this class of receptors (Strader et al., 1994). GPCRs are involved in many important biological functions, such as control of blood pressure, salt and water excretion, neurotransmission, photo- and chemoreception, embryogenesis, angiogenesis and also in pathophysiological processes, such as tumorigenesis and cardiovascular diseases. More than 60% of all drugs currently available act *via* these receptors.

All G protein-coupled receptors have a common structure of seven transmembrane (TM) α-helices, an extracellular N-terminal segment and an intracellular C-terminal segment (see Fig. 2). The transmembrane regions are connected by three extracellular (e1, e2, e3) and three intracellular loops (i1, i2, i3). Each of the seven TM's consists of 20-25 amino acids. The amount of the amino acids of the N terminus, the connecting loops and the C terminus vary considerably. To date, the only member of the receptor superfamily for which a crystal structure has been solved is bovine rhodopsin (Palczewski et al., 2000).
GPCRs can be classified on the basis of sequence homology, ligand structure and ligand binding site into three major families (Fig. 1).

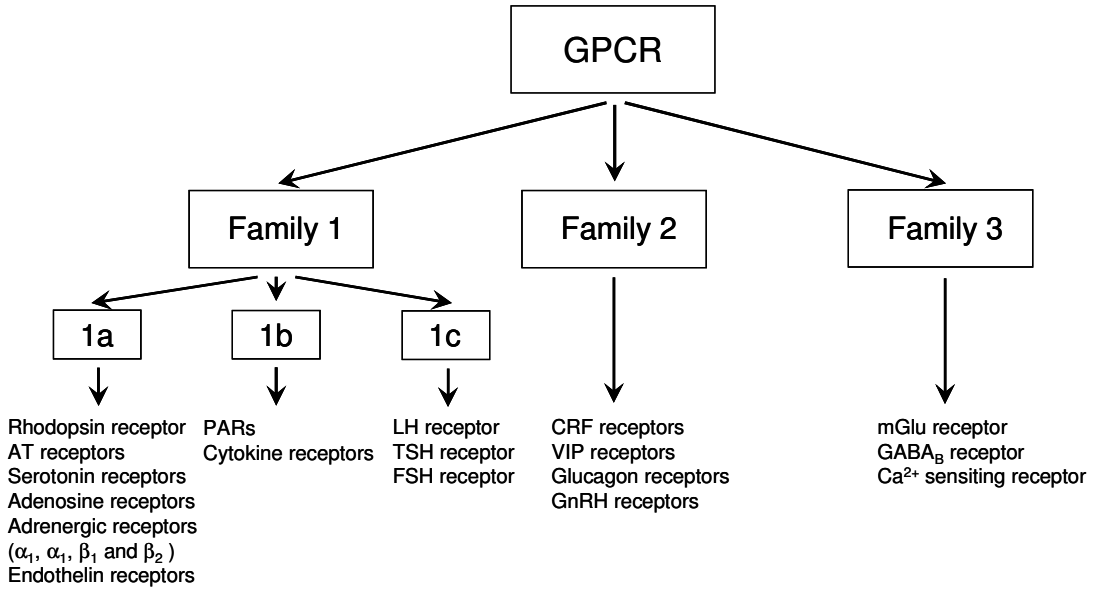

Fig. 1. Classification of G protein-coupled receptors. Examples for each receptor family are presented: AT receptors - angiotensin receptors; PARs – protease-activated receptors; LH receptor - luteinizing hormone receptor; TSH receptor - thyrotrophic stimulating hormone receptor; FSH receptor - follicle stimulating hormone receptor; CRF receptors - corticotropin-releasing factor receptors; VIP receptors - vasoactive intestinal peptide receptors; GnRH receptors - gonadotropin-releasing hormone receptors; mGlu receptor - metabotropic glutamate receptor.

Family 1, or rhodopsin-like family is the largest group and the most studied. It is divided into three subgroups – 1a, 1b and 1c. Also the endothelin receptors belong to this family. Group 1a contains GPCRs for odorants, photons and small molecules such as catecholamines, enkephalins, adenosines. The binding site is localized within the seven TMs. Group 1b contains receptors for peptides whose binding site includes the N terminus, the extracellular loops and the superior parts of TMs. Group 1c contains GPCRs for glycoprotein hormones. The receptors of this family are characterized by a large extracellular domain and a binding site which is mostly extracellular. Family 2 GPCRs have a large N terminus similar to group 1c GPCRs, but they do not share any sequence homology. Their ligands include high molecular weight hormones such as glucagon, secretine and vasoactive intestinal peptide (VIP). Family 3 contains metabotropic glutamate, the Ca^{2+}-sensiting and the γ-aminobutyric acid ($GABA_B$) receptors. These receptors are characterized by a large N terminus of > 400 amino acids. The ligand-binding domain is located in the N terminus.

1.1.1 Signaling of the G protein-coupled receptors

GPCRs are activated by different stimuli and mediate various cellular responses *via* activation of heterotrimeric G proteins. G proteins contain three subunits: α, β and γ. Prior to activation, the α subunit is bound to GDP (see Fig. 2). Upon ligand stimulation of GPCRs, conformational changes in TM3 and TM6 allow binding of heterotrimeric G proteins. This association, in turn, causes conformational changes of the α subunit that lead to the exchange of GDP for GTP and to dissociation of Gα and Gβγ (Bourne et al., 1991). The activated G protein subunits then bind to and regulate various cellular effectors.

In man, at least 16 α, 5 β and 12 γ subunits have been identified (Pierce et al., 2002). Gα subunits can be divided into four families, based on their primary sequence similarity: G_s-, G_i-, $G_{q/11}$- and $G_{12/13}$- family (Wilkie et al., 1992). These G proteins α subunit activate or inhibit effector enzymes that generate specific second messengers. The members of the $G_{q/11}$ family control the activity of phospholipase C isoforms, such as phospholipase $C\beta_{2,3}$ ($PLC\beta_{2,3}$), resulting in the increased formation of intracellular diacylglycerol (DAG) and inositol-1, 4, 5-triphosophosphate (IP_3) (Rhee and Bae, 1997). The α subunit of G_s proteins (stimulatory G proteins) activate adenylyl cyclase (AC), which in turn catalyzes the formation of cAMP from ATP. The α subunit of G_i proteins (inhibitory G proteins) can inhibit certain AC-isoforms (AC V, VI). In addition, released Gβγ dimers are able to regulate the activity of many signaling molecules, e.g. ion channels, phosphatidylinositol 3-kinases (PI3K´s), $PLC\beta_{2,3}$, AC and receptor kinases (Clapham and Neer, 1997). The responses mediated by GPCRs are rapidly turned off when the extracellular signaling ligand is removed. The α subunit is a GTPase which hydrolyzes GTP to GDP. The Gα GDP then dissociates from its effector and reassociates with Gβγ.

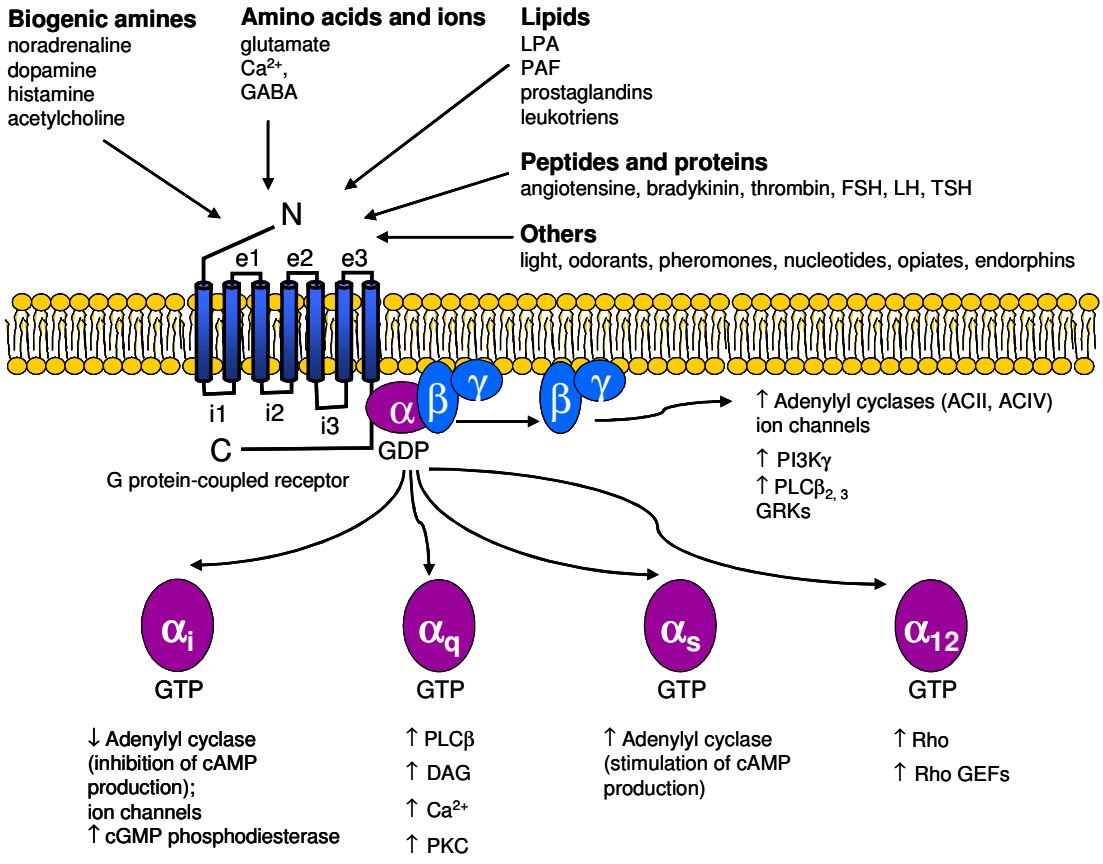

Fig. 2. G protein-coupled receptor activation and downstream signaling. In the absence of agonist, the GPCR is in the low-affinity state. After agonist binding, a transient high-affinity complex of agonist, activated receptor and G protein is formed. GDP is released from the G protein and is replaced by GTP. This leads to dissociation of the G protein complex into α and βγ subunits, which both activate several effectors.

1.1.2 Regulation of G protein-coupled receptor activity

A general property of GPCRs is that in spite of continuing presence of a hormonal ligand, G protein signaling becomes attenuated, which is an adaptive response used by cells to prevent the potentially harmful effects that can result from persistent receptor stimulation (Ostrowski et al., 1992). Three distinct processes contribute to signal attenuation: 1) receptor phosphorylation, 2) receptor internalization, and 3) receptor down-regulation. Receptor phosphorylation, which occurs during short-term (seconds to minutes) exposure to agonists, is mediated by two classes of protein kinases: (i) second messenger-dependent

protein kinases, such as protein kinase A (PKA) and protein kinase C (PKC) and (ii) G protein-coupled receptor kinases (GRKs). Both classes of protein kinases phosphorylate serine and threonine residues within the cytoplasmic third loop and C-terminal tail of GPCRs, leading to increased affinity to β-arrestins. The second messenger-dependent kinases PKA and PKC exhibit the capacity to phosphorylate both agonist-activated receptors and receptors that have not been exposed to agonist. This agonist-independent phosphorylation of GPCRs is termed heterologous desensitization (Lefkowitz, 1993). In contrast, GRKs specifically phosphorylate only agonist-occupied GPCRs. This agonist-dependent phosphorylation is termed homologous desensitization (Inglese et al., 1993; Lefkowitz, 1993; Premont et al., 1995). GRK phosphorylation alone is not sufficient for full receptor desensitization. It has the role to increase the affinity of the receptor for β-arrestins that sterically uncouple the receptor from heterotrimeric G proteins (Lohse et al., 1992).

The internalization of agonist-activated receptors, also termed sequestration, is another mechanism of signal attenuation, which occurs more slowly than phosphorylation. It contributes to receptor desensitization by limiting the number of receptors at the cell surface available for agonist activation. However, receptor internalization is also required for receptor dephosphorylation and resensitization. Internalized GPCRs are targeted to the endosomal compartment of cells where a specific phosphatase dephosphorylates the receptor, followed by β-arrestin dissociation. Subsequently, the receptors are recycled back to the cell surface as fully functional receptors (Bünemann and Hosey, 1999; Carman and Benovic, 1998; Lefkowitz, 1998). The mechanisms involved in the internalization of GPCRs are similar to those involved in receptor desensitization. GPCR endocytosis requires GRK phosphorylation of the cytoplasmic tail of the receptor, followed by its association with β-arrestins, resulting in uncoupling to G proteins. β-arrestins *via* their capacity to interact with both clathrin and the β-adaptin subunit of the AP2 adaptor complex (a complex involved in the formation of clathrin-coated vesicles) target agonist-activated GPCRs to clathrin coated pits. These pits invaginate and form vesicular structures that pinch off from the cell surface and carry the receptor to the endosomal compartment. Two different modes of β-arrestin-mediated internalization are known. Most receptors associate only transiently with β-arrestins. Following endocytosis, β-arrestin dissociates at or near the plasma membrane. Other receptors, such as the vasopressin 2 (V_2) receptor,

internalize as a complex with β-arrestins and traffic with them to the endosomes (Oakley et al., 1999; Oakley et al., 2001). Thus, the formation of a transient GPCR/β-arrestin complex favors rapid dephosphorylation and return to the plasma membrane, whereas the formation of a stable receptor-β-arrestin complex retards resensitization and may favor targeting of the receptor for degradation.

Down-regulation of GPCRs is a persistent reduction of cell surface receptors that occurs during continuous, long-term (hours to days) exposure of cells to agonists. This mechanism of signal attenuation is the least understood process in the control of GPCR function. In response to agonist stimulation some GPCRs, such as protease-activated receptors (PARs), the LH/hCG receptor (Ghinea et al., 1992) and the endothelin B (ET_B) receptor (Oksche et al., 2000a) are internalized but do not recycle. Instead they are sorted to lysosomes and undergo proteolytic degradation (Dery et al., 1999; Hein et al., 1994; Trejo and Coughlin, 1999; Trejo et al., 1998).

Another mechanism, which contributes to receptor down-regulation, is the proteolytic cleavage (limited proteolysis) of the receptor protein at the plasma membrane. Proteolytic processing of GPCRs by cellular proteinases, leading to signal attenuation has been described for the vasopressin V_2 receptor and the β_2-adrenergic receptor. In the case of vasopressin V_2 receptor and the β_2-adrenergic receptor, metalloproteases cleave the activated receptor, thereby promoting down-regulation. V_2 receptor proteolysis occurs rapidly after hormone binding and leads to a loss of ligand-binding properties of the truncated receptor (Kojro and Fahrenholz, 1995). Thus, the proteolytic cleavage of the vasopressin V_2 receptor promotes signaling termination at elevated hormone concentrations.

1.2 Endothelins

The endothelins (ETs) comprise a family of three peptides (ET-1, ET-2 and ET-3) consisting of 21 aa (Fig. 3). They are encoded by distinct ET-related genes located on different chromosomes in the human genome (chromosome 6p23-24 for ET-1, 1p34 for

ET-2 and 20q13.2-13.3 for ET-3) (Inoue et al., 1989). All three members possess two intrachain disulfide bridges, a hydrophobic C-terminal tail and show a high degree of primary amino acid sequence identity (Goldie, 1999; Levin, 1995; Masaki, 2000). These three isoforms also have structural and functional similarities to sarafotoxins, a family of isopeptides isolated from the venom of the snake *Atractaspis engaddensis*, which suggests a possible common evolutionary origin (Takasaki et al., 1988).

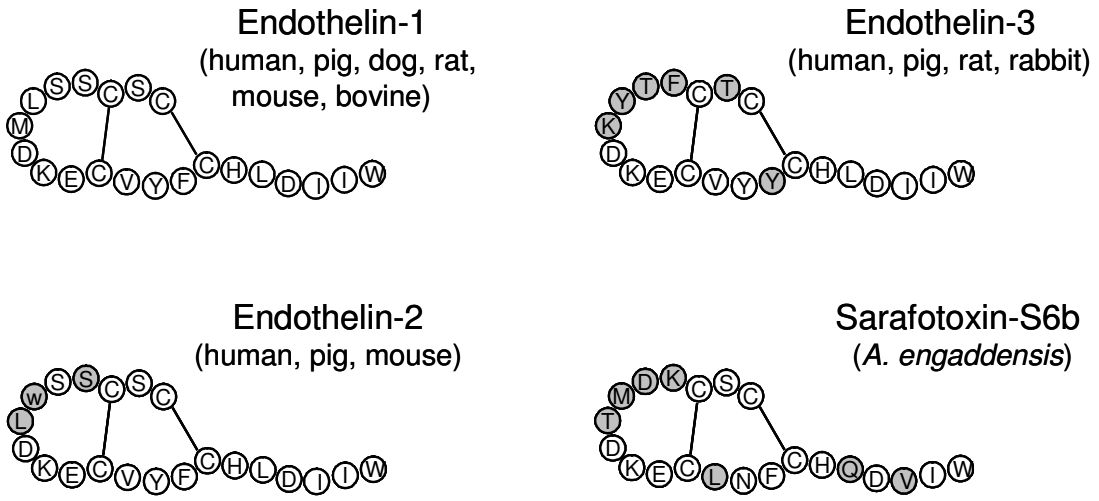

Fig. 3. Primary structures of endothelins and sarafotoxin-S6b. Amino acid residues in ET-2, ET-3 and sarafotoxin-S6b that differ from those in ET-1 are shaded.

ETs are expressed in various tissues and cells (Masaki, 1993). ET-1 is predominantly produced in endothelial cells, but also in epithelial cells, macrophages, fibroblasts, astrocytes, endometrial cells, hepatocytes and cardiomyocytes (Miyauchi and Masaki, 1999). In inflammatory states ET-1 can also be secreted by smooth muscle cells (Tonnessen et al., 1998). ET-2 is produced within the kidney and intestine. ET-3 is found mainly in the brain (Levin, 1995) but also throughout the gastrointestinal tract and in the lung and kidney.

Of the three ET isoforms, the best studied is ET-1. It was identified in 1988 in the supernatant of cultured porcine aortic endothelial cells (Yanagisawa et al., 1988) and is one of the most potent vasoconstrictors known (Levin, 1995). ET-1 is derived from a precursor protein-prepro ET-1 (212 aa) which is cleaved by a furin-like peptidase (Fig. 4). This

generates big-endothelin-1 (big ET-1) consisting of 39 amino acids, which is biologically inactive. Subsequently, a specific endothelin-converting-enzyme-1 (ECE-1) cleaves big ET-1 between Trp21 and Val22, thereby producing mature ET-1 (Valdenaire et al., 1995). ECE-1 is classified as a membrane-bound metalloprotease, which is phosphoramidon-sensitive.

About 80 % of the ET-1 is secreted at the abluminal membrane of endothelial cells, toward the smooth-muscle layer of the wall of the blood vessel, where it can bind to specific ET receptors on the vascular smooth muscle cells (VSMCs). A smaller amount of the peptide is secreted into the lumen of the vessel (the plasma concentration of ET-1 is approximately 1 fmol/ml) (Wagner et al., 1992). Therefore, ET-1 is regarded more as a paracrine than as an endocrine hormone.

There is little intracellular storage of ET-1. The peptide production is regulated at the level of gene transcription with de novo synthesis of the precursor prepro ET-1 (Wilson et al., 1990). Numerous factors stimulate ET-1 production, which can be classified into broad categories of vasoconstrictor/thrombogenics such as angiotensin II (Ang II), adrenalin, thrombin, prostaglandin $F_{2\alpha}$ and ET-1 itself; inflammatory cytokines, such as interleukin-2 (IL-2) and transforming growth factor-β (TGF-β); physical factors, such as mechanical strain, pressure and shear stress; growth factors, such as epidermal growth factor (EGF) and basic fibroblast growth factors; insulin and hypoxia (Fig. 4). ET-1 synthesis is down-regulated by vasodilators/anticoagulants, e.g. prostacyclin, prostaglandin E_2, nitric oxid (NO), atrial natriuretic peptide and heparin (Boulanger and Lüscher, 1990; Hu et al., 1992; Prins et al., 1994).

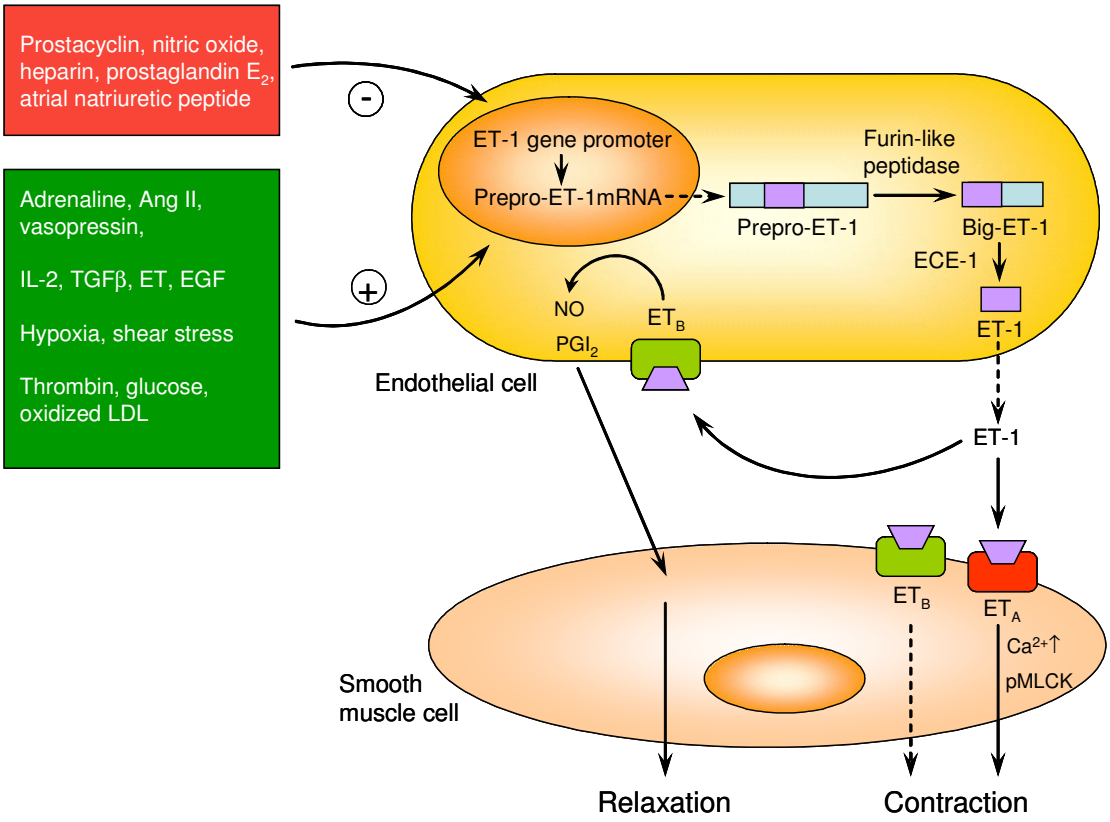

Fig. 4. Biosynthesis of ET-1, its regulation and ET receptor-mediated actions on smooth muscle cells. ET-1 synthesis is regulated by many factors; stimulators are highlighted in green, and the inhibitors are highlighted in red. ET-1 is produced from a precursor, named preproendothelin. After the removal of a signal peptide, the precursor is selectively processed by an enzyme (a furin-like peptidase to yield a biologically inactive intermediate called big-endothelin-1 (big ET-1). Big ET-1 is further converted into active ET by endothelin-converting enzyme-1 (ECE-1). Two ET receptors have been identified: ET_A receptors are predominantly expressed on vascular smooth muscle cells and mediate vasoconstriction, whereas ET_B receptor are mainly found on endothelial cells and induce vasodilation through NO. ET_B receptors on smooth muscle cells can elicit vessel contraction.

The ETs have a variety of biological activities such as vasoconstriction, bronchoconstriction, uterine smooth muscle contraction, vasorelaxation, inhibition of platelet aggregation and stimulation of aldosterone and natriuretic hormone secretion (see tab.1) (Miyauchi and Masaki, 1999). ET-1 may mediate the effects of several vasoactive peptides and growth factors. For instance, Ang II stimulates cardiac hypertrophy (Sadoshima et al., 1993) and vascular proliferation. Inhibition of either Ang II-stimulated ET-1 production or the action of ET-1 can prevent the cardiac hypertrophic effects of Ang

II (Ito et al., 1993). In turn, ET-1 can stimulate the conversion of Ang I to Ang II and potentiate the Ang II-induced production of aldosterone (Cozza et al., 1992). The interactions between these two hormones are potentially important in view of the strong evidence that angiotensin-converting-enzyme inhibitors prolong survival in patients with left ventricular dysfunction after myocardial infarction (Pfeffer et al., 1992).

1.3 Endothelin receptors

ETs exert their effects *via* endothelin A (ET_A) and endothelin B (ET_B) receptors (Arai et al., 1990; Sakurai et al., 1990). In man, the ET_A and the ET_B receptor genes are located on chromosome 4 and 13, respectively. Both receptors belong to the G protein-coupled receptor system (family 1a) and share 64% aa sequence identity. The ET receptor subtypes show differences in their tissue distribution, ligand binding, signal transduction and mode of internalization.

ET_A receptors are mainly expressed in smooth muscle cells of the vascular medial layer and the bronchiole (Chen et al., 2003). ET_B receptors are predominantly expressed in endothelial cells and in small amounts in VSMCs (Maguire and Davenport, 1995). They are also expressed in the renal proximal and distal tubule, the renal collecting duct and in the cells of the atrioventricular conducting system. In addition, ET_A and ET_B receptors are co-expressed in numerous cells, e.g. astrocytes, cardiomyocytes, epithelial cells of the choroids plexus and the anterior pituitary (Angelova et al., 1997; Harada et al., 2002; Kitsukawa et al., 1994). In disease states, such as atherosclerosis and hypercholesterolemia, ET_B receptors are upregulated in VSMCs of vessels with atherosclerotic lesions (Hansen-Schwartz, 2004; Iwasa et al., 1999; Sihvola et al., 2002; Wackenfors et al., 2004).

ETs form with their receptors stable complexes which dissociate very slowly under physiological conditions (Clozel et al., 1989; Fischli et al., 1989; Martin et al., 1989). The ET_A receptor binds ET-1 and ET-2 with the same and ET-3 with lower affinity (ET-1=ET-2>>ET-3), whereas the ET_B receptor binds all three isoforms with equal affinity (ET-1=ET-2=ET-3) (Masaki, 2004).

The ET_B receptor binds ET-1 almost irreversibly (Waggoner et al., 1992). The ET-1/ET_B receptor complex is resistant to acid washes and 2% SDS and remains stable in SDS-polyacrylamide gel electrophoresis at low temperature (Akiyama et al., 1992). In living cells, internalized receptor-ligand complexes remain stable for more than 2 h, even after their sorting to the late endosomal/lysosomal compartments (Oksche et al., 2000a; Oksche et al., 2000b). Mutagenesis studies revealed that aspartate 75 and proline 93 within the extracellular N terminus of the human ET_B receptor are important determinants for the formation of a stable ligand/receptor complex (Takasuka et al., 1994). The physiological significance of this tight receptor/ligand association is still not understood. Recently, it was shown that the ET_B receptor is crucially involved in the regulation of circulating ET-1 plasma levels: blockade of the ET_B receptor causes a significant increase in ET-1 plasma levels (Dupuis et al., 1996; Fukuroda et al., 1994). Thus, the tight binding of ET-1 to the ET_B receptor and the transport of the receptor/ligand complex into lysosomal compartments could provide a molecular basis for the efficient removal of ET-1 from the circulation (scavenger function).

Another unique feature of the ET_B receptor, which is not shared by the ET_A receptor, is the limited proteolysis of the extracellular N terminus. Both ET receptors posses a cleavable signal peptide which is essential for the cell surface transport: for the human ET_A receptor the first 16 aa function as a signal peptide and for the human ET_B receptor the first 26 aa (see Fig. 5). The signal peptide is cleaved off by a signal peptidase in the ER lumen during receptor biosynthesis (Akiyama et al., 1992; Köchl et al., 2002; Saito et al., 1991). Only for the ET_B receptor a second proteolytic cleavage within the extracellular N terminus has been demonstrated (Akiyama et al., 1992). This cleavage occurs 38 amino acids C-terminally of the signal peptidase cleavage site, between arginine 64 and serine 65 (R64/S65). The proteolytically released N-terminal peptide fragment harbors the only N-linked glycosylation (N59/A60/S61). Thus, the N-terminal proteolysis results in a truncated, non-glycosylated receptor.

The existence of this N-terminal cleavage has been demonstrated also for the dog, pig and calf receptor, indicating that the proteolytical processing of the ET_B receptor is evolutionally conserved (Hagiwara et al., 1991; Saito et al., 1991; Takasuka et al., 1991; Takayanagi et al., 1991). However, the mechanism and physiological significance of the N-terminal proteolysis for this receptor are unknown.

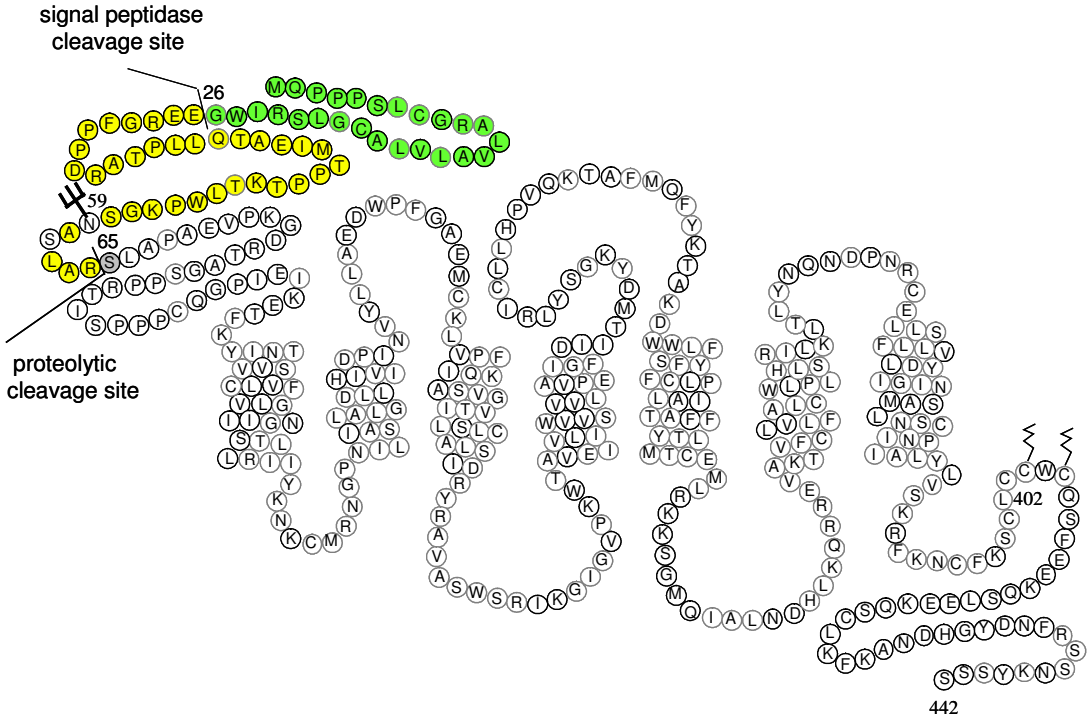

Fig. 5. Scheme of the human ET_B receptor. The first 26 aa represent a signal peptide which is cleaved off by a signal peptidase within the ER lumen. A further cleavage of the N terminus occurs at 64 aa. Asp^{59} is the N-glycosylation site and Cys^{402} and Cys^{404} are palmitoylation sites.

1.3.1 Signal transduction of endothelin A and endothelin B receptors

Both endothelin receptor subtypes couple to diverse G proteins, stimulating thereby different signaling cascades. The ET_A receptor activates G proteins of the $G_{q/11}$ and $G_{12/13}$ family (Takuwa et al., 1990) and the ET_B stimulates G proteins of the G_i and $G_{q/11}$ family (Aramori and Nakanishi, 1992; Eguchi et al., 1993a). Both receptors activate PLCβ *via* $G_{q/11}$ proteins and βγ subunit, which stimulate the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), leading to the formation of inositol-1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG) (Sokolovsky, 1995) (Fig.6).

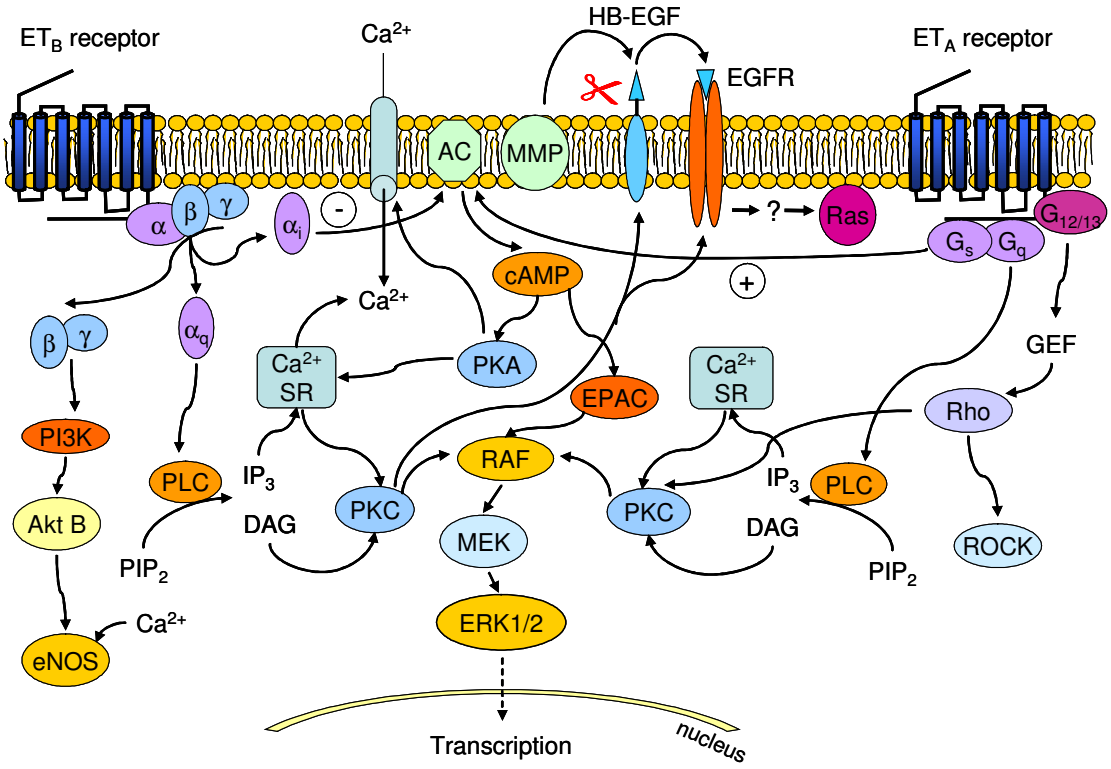

Fig. 6. Signal transduction of the human ET_A and ET_B receptors. AC – adenylyl cyclase; MMP – matrix metalloproteases; EGFR – epidermal growth factor receptor; HB-EGF – heparin-binding epidermal growth factor; PKA – protein kinase A; PKC – protein kinase C; cAMP – cyclic AMP; PLC - phospholipase C; DAG - 1,2-diacylglycerol; PIP_2 – phosphatidylinositol-4,5-bisphosphate; IP_3 – inositol-1,4,5-triphosphate; PI3K - phosphatidylinositol-3-OH kinase; eNOS - endothelial NO-synthase; Akt B – protein kinase B; ERK – extracellular-signal regulated kinase; GEF – guanine nucleotide exchange factor; ROCK – RhoA kinase; MEK – mitogen-activated protein kinase (MAPK) kinase.

IP_3 stimulates the release of Ca^{2+} from intracellular stores which results in a calmodulin-dependent activation of the myosin light chain kinase (MLCK). In turn MLCK phosphorylates the 20 kDa myosin-light chain (MLC-20) which promotes actin-myosin interactions in VSMCs, resulting in vasoconstriction. In addition, DAG and Ca^{2+} stimulate PKC (Nishizuka, 1992), leading to the activation of the MAPK signaling pathway (Cazaubon et al., 1993; Wang et al., 1992).

The ET_A receptor activates Rho *via* $G_{12/13}$. Rho is a small GTPase involved in a wide range of cellular processes (Gohla et al., 2000). In VSMCs stimulation of Rho causes Rho-kinase

activation, which inhibits the myosin-light chain phosphatase. Thus, dephosphorylation of MLC-20 is inhibited, thereby leading to a prolonged constrictory response.

In endothelial cells, the ET_B receptor stimulates the release of the NO and prostacyclin (PGI_2) *via* pertussis toxin (PTX)-sensitive G proteins, causing relaxation of VSMCs and subsequent vasodilation. NO is produced from endothelial NO-synthase (eNOS) *via* G_i-mediated increase in cytosolic Ca^{2+} concentration and G_i βγ-mediated activation of Akt/PKB. Prostacyclins (PGI_2) are generated by an increased release of arachidonic acid *via* phospholipase A2 and the action of cyclooxygenase 2 (COX 2) (Resink et al., 1990). In addition, the ET_B receptor also inhibits the cAMP formation (Aramori and Nakanishi, 1992; Eguchi et al., 1993a). This signaling step is of importance in the differentiation of astrocytes.

The ET_A and ET_B receptors can activate MAPK through two independent signaling pathways: through a PKC-dependent pathway, involving the transactivation of EGF receptor and through phosphatidylinositol-3-OH kinase (PI3K) dependent pathway (Eguchi et al., 1998; Iwasaki et al., 1999). In addition, ET_B receptor can stimulate MAPK through a PTX-sensitive pathway which involves the activation of the small GTPase Ras and EGF receptor (Daub et al., 1996). However, the steps between the ET_B and EGF receptor, and, on the other hand, between the EGF receptor and ERK 1/2 remain elusive. The significance of this signaling pathway is also not known yet.

1.3.2 Internalization and down-regulation of the endothelin receptors

Upon ligand binding, both endothelin receptor subtypes are rapidly desensitized by phosphorylation of the C-terminal tail through the G protein-coupled receptor kinase type 2 (GRK2) (Freedman et al., 1997). However, desensitization is also observed for the ET_B receptor coexpressed with a catalytically inactive GRK2 (K220W/GRK2) and for a mutant ET_B receptor lacking the C-terminal 40 amino acids (Shibasaki et al., 1999). Thus other mechanisms might contribute to the desensitization of ET_B receptors.

The endothelin receptor subtypes differ in their mode of internalization. Upon agonist stimulation both receptor subtypes are rapidly internalized and initially appear to share a

common path into Rab5-positive early endosomes. However, the two receptor subtypes are subsequently targeted to different intracellular compartments. The ET_A receptor follows the recycling pathway, in which it is directed to recycling endosomes and subsequently reappears at the plasma membrane (Bremnes et al., 2000; Okamoto et al., 2000). In contrast, the ET_B receptor is transported to late endosomes/lysosomes for degradation (Bremnes et al., 2000; Oksche et al., 2000a). The signals for the ET_B receptor internalization and for endosomal/lysosomal sorting reside in the C-terminal tail of the receptor (Paasche et al., 2001).

1.4 Physiological role of the endothelin system

ETs mediate their vasoconstrictor action *via* ET_A receptor: its stimulation in VSMCs induces long-lasting vasoconstriction *via* an increase in intracellular Ca^{2+} (Seo et al., 1994). The vasoconstriction persist after ET-1 is removed from the receptor (Clarke et al., 1989). Thus, it is supposed that ET-1 and ET_A receptor play a role in the maintenance of basal vasomotor tone. NO shortens the duration of vasoconstriction by accelerating the return of intracellular Ca^{2+} to its basal concentration (Goligorsky et al., 1994). The activation of the ET_B receptor expressed in endothelial cells mediates the release of NO and prostacyclin thereby causing transient vasodilation (de Nucci et al., 1988). In the kidney, ET_A receptors which are predominantly expressed in the vasa recta and arcuate arteries, regulate the renal circulation (Ferrario et al., 1989), whereas ET_B receptors are involved in natriuresis and diuresis. The natriuretic action occurs *via* the inhibition of the amiloride-sensitive sodium channel (ENaC). In the lung, ET_A receptors mediate pulmonary vasoconstriction and ET_B receptors - bronchoconstriction. In astrocytes ET_B receptors mediate the ability of the ET-1 and ET-3 to stimulate the synthesis of DNA and to inhibit the generation of second messengers by other neurotransmitters (Levin et al., 1992).

Tissue/organ	Cell source of ET	Physiological effects of ET	ET receptor	References
Vasculature	• Endothelial	• Transient vasodilation • Sustained vasoconstriction	ET_B ET_A	*Yanagisawa et al., 1988*
Heart	• Endothelial • Smooth muscle • Cardiomyocytes • Fibroblasts	• Increased coronary vascular resistance • Decreased cardiac output • Hypertrophy of cardiomyocytes • Hypertrophy/proliferation of fibroblasts	ET_A ET_A ET_A ET_A, ET_B	*Miller et al., 1989* *Ito et al.,1991* *Spieker et al., 2001*
Kidney	• Endothelial • Smooth muscle • Mesangial	• Renal vasoconstriction • Increased glomerular afferent and efferent arteriolar tone • Mesangial-cell proliferation • Natriuresis, diuresis • Increased Na transport in proximal tubule	ET_A ET_A ET_A, ET_B ET_A, ET_B ET_A, ET_B	*King et al., 1989* *Edwards et al., 1990* *Badr et al., 1989* *Ischimura et al., 1991*
Lung	• Endothelial • Airway epithelial	• Pumonary artery constriction • Contraction of airway smooth muscle • Mucous-gland hypersecretion	ET_A ET_B ET_A, ET_B	*Fagan et al., 2001* *Goldie and Fernandes, 2000*
Brain	• Endothelial • Neurons • Astrocytes	• Constriction of cerebral arteries • Activation of Na/K/Cl-transporter function on brain capillary endothelial cells • Inhibition of generation of second messengers by neurotransmitter cathecolamines	ET_A ET_A (?), ET_B ET_B	*Encabo et al., 1992* *Vigne et al., 1994* *Levin et al., 1992*

Tab. 1. Biological actions of ET on different tissues and organs.

1.5 The role of the endothelin system in disease

ETs play crucial role in numerous diseases, such as chronic heart failure, ischemic heart disease, hypertension, atherosclerosis, pulmonary hypertension, chronic renal failure and cerebrovascular spasm after subarachnoid hemorrhage.

Ischemic heart diseases and chronic heart failure

ET-1 plays an important pathogenic role in the myocardial damage that follows acute ischemia. Plasma ET-1 concentrations are increased after myocardial infarction in animal models and humans (Battistini et al., 1993). Intravenous infusion of ET-1 reduces coronary blood flow more than 90 % (Kurihara et al., 1989). In dogs, infusion of the ET_A receptor antagonist BQ123 reduces the extent of experimentally induced myocardial infarction by 40 % (Grover et al., 1993).

Plasma ET-1 levels are elevated in both humans (Hiroe et al., 1991; McMurray et al., 1992; Stewart et al., 1992) and experimental animal models (Margulies et al., 1990; Underwood et al., 1992) with chronic heart failure. To compensate for decreased cardiac function, the ET system is upregulated. However, sustained activation of the ET system might result in the deterioration of cardiac function, causing, for example, arrhythmia and cardiac hypertrophy.

Hypertension

The importance of the ET system in the pathogenesis of essential hypertension remains controversial. ET_A receptor antagonists BQ123 and darusentan have been shown to decrease selectively arterial pressure in animal models. Genetic and pharmacological disruptions of the ET_B receptor in mice elevate systemic blood pressure, which is linked to the ET_B receptor activation of prostaglandins (Grover et al., 1993). Significant lowering of blood pressure has been reported in a systemic study of bosentan (ET_A and ET_B receptor antagonist) and darusentan in hypertensive patients (Krum et al., 1998; Nakov et al., 2002).

Since ETs and ET receptors are expressed in various kidney cells and play important role in renal hemodynamics and urinary salt and water excretion, an involvement of the ET system in renal hypertension is suggested.

As ET-1 is known to be produced by amniotic and endometrial cells, it could contribute to the hypertension that characterizes preeclampsia. Plasma ET-1 concentrations are increased in women with preeclampsia (Branch et al., 1991).

Pulmonary hypertension

Pulmonary hypertension is characterized by an increase in pulmonary vascular tone, vasoreactivity and enhanced proliferation of bronchial VSMCs, resulting in increased pulmonary vascular resistance and right ventricular hypertrophy (Heath et al., 1987). Pulmonary hypertension is associated with elevated plasma ET levels (Cernacek and Stewart, 1989) and the plasma concentration of ET-1 correlates with the degree of the disease (Cacoub et al., 1993; Ishikawa et al., 1995; Yoshibayashi et al., 1991). In patients with pulmonary hypertension the expression of ET-1 mRNA, ET-1 and ET_A receptors in pulmonary vascular endothelial cells is enhanced (Giaid et al., 1993b), suggesting that local production of ET-1 may contribute to the vascular abnormalities associated with this disorder. Similar observations have been made in patients with cryptogenic fibrosing alveolitis (Giaid et al., 1993a). In addition, ET_A and ET_B receptor antagonism with bosentan has been shown to improve specifically the pathological increase in pulmonary arterial pressure (Channick et al., 2001; Rubin et al., 2002).

Renal failure

ET-1 contributes to several vascular-related diseases of the kidney. Increased ET-1 gene expression and peptide production have been shown to occur in response to a variety of injurious stimuli, including ischemia, radiocontrasts, proteinuria, cyclosporine and other nephro-toxic agents (Heyman et al., 1992; Kohan, 1997). Moreover, ET antagonism improves renal failure even after acute renal failure is well established (Gellai et al., 1994; Kon et al., 1989). Increased ligand binding affinity of both the ET_A and ET_B receptors in the renal cortex follows ischemia. This effect is more pronounced in the medulla than in the cortex and affects ET_B receptors more than ET_A receptors. ET_B, but not ET_A, mRNA is also elevated in a renal post-ischemic model, and again is particularly apparent in the medulla. Nephrotoxicity from cyclosporine also preferentially increases the ET_B receptor in the renal medulla (Iwasaki et al., 1994; Takeda et al., 1992). Thus, ischemia and nephrotoxic injury particularly affect the ET_B receptor and the renal medulla. ETs also

participate in all the processes known to be involved in renal parenchymal destruction of chronic renal failure, including cellular hypertrophy, proliferation and collagen deposition (Hocher et al., 1997).

Developmental disorders

Mice homozygous for ET-1 or ET_A receptor gene disruption have severe malformations of craniofacial tissues derived from the first pharyngeal arch, such as cleft palate, micrognathia, microtia and microglossia. ET_A (-/-) or ET-1 (-/-) mice die of respiratory failure at birth (Kurihara et al., 1994).

The activation of ET_B receptor by ET-3 is critical for the normal development of cells derived from neural-crest precursors (Baynash et al., 1994). Epidermal melanocytes and ganglionic neurons in the intestine do not develop when the ET_B receptor functions improperly or when ET-3 is not produced. Mice with a disruption of ET-3 or the ET_B receptor gene display pigment disorder (white spotting of their coats) and aganglionic megacolon (Hirschsprung´s disease) (Hosoda et al., 1994). A hereditary form of Hirschprung´s disease in humans has also been found to be associated with several different mutations of the gene for the ET_B receptor and ET-3 (Puffenberger et al., 1994). The lack of ET-3/ET_B receptor results in the absence of parasympathic ganglionic neurons in the myenteric plexus (Auerbach).

1.6 The aim of this study

ET_B receptors of different species display an N-terminal signal peptide of 26 amino acids which is cleaved off in the lumen of the endoplasmic reticulum. The presence of the signal peptide is essential for the cell surface transport as a mutant ET_B receptor lacking this N-terminal signal sequence is retained within the cell (Köchl et al., 2002). A second proteolytic cleavage site within the N terminus of the ET_B receptor has been reported, resulting in a truncated receptor lacking further 38 amino acids C-terminal of the signal peptidase cleavage site. The existence of two different ET_B receptor isoforms has been demonstrated in membrane preparations of bovine lung, human placenta, canine and porcine heart (Takasuka et al., 1991), as well as for human and bovine ET_B receptors transiently or stably expressed in Sf9, COS1 and CHO cells (Takasuka et al., 1994; Hagiwara et al., 1991; Satoh et al., 1997; Oksche et al., 2000a). It remains elusive whether both isoforms are expressed at the cell surface of intact cells or are due to receptor degradation following the release of proteases during the membrane preparation. To date, the physiological significance of this N-terminal proteolysis is unknown and the functional properties of the truncated receptor have not been studied so far. This is, however, of interest, as N-terminal proteolysis of the ET_B receptor might be involved in the regulation of receptor signaling and/or receptor internalization.

The aim of the present study was to characterize which ET_B receptor isoforms are expressed in intact cells. In addition, the mechanisms underlying the N-terminal proteolysis and the modes of internalization and signal transduction of the two receptor isoforms are studied.