

4 Discussion

4.1 The short isoform of the human ET_B receptor is generated by a proteolytic cleavage upon agonist stimulation

Protein analysis of the ET_B receptor in membrane preparations of human placenta revealed two different isoforms: i) a full-length receptor comprising 416 amino acids (after removal of the signal peptide) and starting with glutamate 27, and ii) an N-terminally truncated ET_B receptor, comprising 378 amino acids and starting with serine 65 (Akiyama et al., 1992; Satoh et al., 1997). However, it was not known whether both receptor isoforms are expressed at the cell surface or are the result of receptor degradation following the release of proteases during membrane preparation.

LT-PAGE analyses demonstrated that the ET_B receptor is expressed as a full-length receptor at the cell surface of intact HEK 293 cells and VSMCs and that the N-terminal proteolysis is induced by binding of ET-1 to the receptor (see Fig. 9 and Fig. 10). The N-terminal cleavage was visible after 15 min and was almost complete within 60 min of agonist application (Fig. 13). The ligand-induced cleavage was also observed in the presence of sucrose (an inhibitor of clathrin-mediated endocytosis), phenylarsine oxide (inhibits clathrin-dependent and -independent endocytosis) and leupeptin (an inhibitor of lysosomal cysteinyl proteases), indicating that the N-terminal proteolysis does not depend on receptor internalization and occurs at the plasma membrane. The finding that the cleavage is promoted upon binding of the receptor agonist was confirmed by Western blot (see Fig. 11) and pulse-chase experiments (see Fig. 12), allowing to detect the native ET_B receptor without prior exposition to the radioactively labeled ET-1.

4.2 *N-terminal proteolysis is mediated via a metalloprotease*

The proteolytic cleavage was inhibited in the presence of metal chelators, such as EDTA and phenantroline, and metalloprotease inhibitors (batimastat and TACE inhibitor Ro 32-7315), which points to a role of metalloproteases in the N-terminal proteolysis. Batimastat and Ro 32-7315 block a variety of different metalloproteases, including MMPs and members of a disintegrin and metalloprotease (ADAM) family (Gomis-Ruth et al., 1998; Maskos et al., 1998; Wojtowicz-Praga et al., 1997). Specific metalloprotease inhibitors are only available for gelatinases A and B (MMP-2 and MMP-9 respectively). Ro 28-2653, which inhibits gelatinase A and B did not prevent N-terminal proteolysis, indicating that these types of metalloproteases are not involved. Thus, the exact identity of the metalloprotease involved remains elusive.

The inhibitory effect of EDTA, batimastat and Ro 32-7315 were most effective when the internalization was inhibited by the addition of sucrose or phenylarsine oxide. Considering the fact that the ET_B receptor is quantitatively removed from the cell surface by internalization within 30 min (Oksche et al., 2000a) and that the N-terminal proteolysis requires about 60 min (this study), it is likely that the metalloprotease is internalized together with the receptor/ligand complex (compare Fig. 13 and Fig. 15). It may be possible that during endocytosis the inhibitors have a reduced activity due to the acidic pH of endosomes. Another explanation could be that the metalloprotease inhibitors and receptor/ligand complexes are sorted to different destinations within the cell, resulting in a dilution of the inhibitor.

4.3 *Specificity of N-terminal cleavage site*

It is notable that site-directed mutagenesis of the putative cleavage site R64↓S65 did not prevent the proteolytic processing of the N terminus. Curiously, in the mutant HIS ET_B receptor even the exchange of 9 amino acids did not prevent N-terminal proteolysis (see Fig. 16). Thus, the cleavage appears to be independent of the primary sequence. This result is in line with several reports on the proteolysis of integral membrane proteins by metalloproteases. For example, the amyloid precursor protein (APP) is cleaved by the α -

secretase 12 amino acids distal of the transmembrane domain (K16↓L17). The cleavage still occurs, when the cleavage site is mutated or deleted. Further studies revealed a dependence of the cleavage on an α -helical conformation and the distance of the cleavage site from the plasma membrane (Esch et al., 1990; Sisodia, 1992). L-selectin cleavage (K321↓S322) also depended on the distance of the cleavage site from the plasma membrane, but not on the amino acid sequence (Hooper et al., 1997; Schlöndorff and Blobel, 1999). Therefore, it is possible, that the metalloprotease cleaves the ET_B receptor in an unfolded region of the N terminus with a certain distance to the plasma membrane, independent from the amino acid sequence. Due to a lack of the structural information of the ET_B receptor or its N terminus, it is unknown, whether the region surrounding the putative proteolytic cleavage site R64↓S65 of the N terminus is unfolded. For the purified ET_B receptor, however, it has been demonstrated that this region is accessible for proteases. Treatment of the ET_B receptor (displays 18 arginine residues) with the endopeptidase Arg-C resulted in the exclusive cleavage after R64. Similarly, thrombin and thermolysin were found to cleave the ET_B receptor at R64 (Wada et al., 2001).

4.4 Functional consequences of the N-terminal cleavage

N-terminal proteolysis of the ET_B receptor could result in a receptor with altered pharmacological properties. Proteolysis could change the pattern of activated G proteins, modulate receptor internalization or alter the affinity for different ligands. Saturation binding experiments of transiently transfected cells revealed normal affinity to ET-1 but a 15-fold lower cell surface expression for the Δ 2-64 ET_B receptor, than for the wild-type receptor. The mutant HIS ET_B receptor with an altered cleavage site and an abolished N-linked glycosylation showed similar expression levels as the full-length receptor. Thus, the N terminus seems to be of importance for proper cell surface expression of the ET_B receptor, whereas the lack of N-linked glycosylation has no significant effects on the receptor numbers at the cell surface. It is possible that the N-terminal cleavage causes a conformational change, leading to a more efficient interaction with proteins regulating cell surface expression (e.g. adapter proteins). However, reduced receptor numbers at the cell

surface could also be attributed to a less efficient synthesis of the $\Delta 2-64$ ET_B receptor, since it additionally lacks a cleavable signal peptide.

The full-length ET_B receptor and the N-terminally truncated $\Delta 2-64$ ET_B receptor mutant showed similar modes of internalization. These findings indicate that the N-terminal proteolysis did not alter the ability of the ET_B receptor to internalize upon ligand stimulation.

No differences in the G protein signaling of the wild-type and the truncated ET_B receptor were observed. Both receptors stimulated inositol phosphate formation (predominantly mediated through G_{q/11}) and decreased the activity of the adenylyl cyclase (mediated through G_i) to the same extent, indicating that the proteolytic cleavage did not alter the ability of the ET_B receptor to activate G proteins of G_{q/11} and G_i families. Thus, no difference in the pattern of the activated G proteins was observed.

4.4.1 The full-length ET_B and the $\Delta 2-64$ ET_B receptors differ in their ability to induce ERK1/2 activation

Despite the fact that both full-length and N-terminally truncated ET_B receptors stimulate G_{q/11} and G_i proteins, the two receptor isoforms showed differences in ERK1/2 activation. Both receptors induced activation of ERK1/2 for up to 40 min, which was in agreement with previous studies, demonstrating that the ET_B receptor stimulates ERK1/2 phosphorylation up to 40 min *via* G proteins of G_{q/11} family (Arai et al., 2003; Cramer et al., 2001; Schlöndorff and Blobel, 1999). Strikingly, for the full-length receptor with an intact N terminus a long-lasting second phase of ERK1/2 phosphorylation was observed (see Fig. 18A and B). Further experiments revealed that the second phase is mediated *via* $\beta\gamma$ subunit of G_i proteins. Thus, the full-length ET_B receptor stimulates the first and second phase of ERK1/2 activation *via* two different signaling cascades, e.g. a PTX-insensitive and PTX-sensitive signaling cascade, respectively. This biphasic ERK1/2 activation was not found for the N-terminally truncated $\Delta 2-64$ ET_B receptor, although the $\Delta 2-64$ and the full-length ET_B receptor stimulated both G_i and G_{q/11} proteins. The reason for the differences in the pattern of ERK1/2 activation between both receptor isoforms remains

elusive. It is possible, that the intact N terminus or the N-terminal proteolysis are involved in the generation of a biphasic ERK1/2 activation.

The functional significance of the observed biphasic ERK1/2 phosphorylation is unclear. It is notable that in many cells (e.g. PC12 cells and VSMCs) the intensity and the duration of ERK1/2 activation determine the cellular response, e.g. proliferation or differentiation (Kao et al., 2001; York et al., 1998; Zhang et al., 2000). While a monophasic, short-term activation of ERK1/2 (~30 min) *via* the EGF receptor results in proliferation, a biphasic ERK1/2 activation *via* NGF / trkA activation was found to mediate differentiation. Similarly, stimulation of PAR1 in VSMCs has been reported to induce differentiation through PTX-sensitive biphasic ERK1/2 activation (Reusch et al., 2001). Thus, it is possible that the ET_B receptor also stimulates differentiation of cells. The N-terminally truncated Δ 2-64 ET_B receptor, which induced a transient, monophasic ERK1/2 activation, should possess mitogenic properties only.

4.5 Limited proteolysis in other G protein-coupled receptors

The significance of the N-terminal cleavage for the ET_B receptor down-regulation remains unclear. Since the cell surface expression of the truncated Δ 2-64 ET_B receptor mutant is dramatically reduced, the N-terminal proteolysis could be involved in the down-regulation of the ET_B receptor. The ligand-dependence of the cleavage favors this postulation. Moreover, proteolytic cleavage of GPCRs after agonist stimulation at the plasma membrane has been shown to regulate the functional state and the number of receptors at the cell surface. A limited proteolysis has been demonstrated for the vasopressin V₂ receptor, the β ₂-adrenergic receptor and the thyrotrophic stimulating hormone receptor TSH receptor. In the case of the vasopressin V₂ receptor a metalloprotease specifically cleaves the ligand-bound receptor between glutamine 92 and valine 93 within the second transmembrane domain (Kojro et al., 1999), leading to a loss of ligand-binding properties. In the β ₂-adrenergic receptor, metalloproteases-mediated proteolysis occurs after continuous stimulation for more than six hours and promotes down-regulation (Jockers et al., 1999). The cleavage site within the β ₂-adrenergic receptor has not been determined. Thus, the limited proteolysis of both GPCRs is likely to fulfill two different functions: in

the case of the vasopressin V_2 receptor it could be involved in the short-term regulation of the receptor and in the case of the β_2 -adrenergic receptor-in the long-term down-regulation. In the TSH receptor, spontaneous release of the highly glycosylated extracellular N terminus was observed (Couet et al., 1996). The release was promoted slightly by TSH, phorbol esters and forskolin. Its physiological importance is not yet understood.

Recently, it has been demonstrated that metalloproteases can cleave the N terminus of the protease-activated receptor 1 (PAR1) proximal to the thrombin cleavage site, resulting in a non-functional receptor (Ludeman et al., 2004). In contrast, the N-terminal proteolysis of the ET_B receptor, does not destroy receptor function and the overall structure. However, the N-terminal proteolysis of the ET_B receptor shows some similarities to the PARs, for which a generation of a “tethered ligand” was found. In the case of PARs, the serine proteases thrombin (PAR1, PAR3) and trypsin (PAR2) cleave a portion of the receptor’s N terminus, unmasking a new amino-terminal peptide. This peptide activates the receptor irreversibly, promoting its endocytosis. Subsequent sorting to lysosomes and degradation was thought to represent the only efficient mechanism to prevent continuous activation (Dery et al., 1999; Trejo and Coughlin, 1999). Interestingly, the new N terminus of the truncated ET_B receptor (38 amino acids) is similar in length as those of the PAR family (about 23 to 72 amino acids). However, the new N terminus (SLAPAE) of the ET_B receptor does not share sequence similarity with that of PAR1, PAR2 and PAR4 receptors (SFLLRN/TFLLR, SLIGRL, and GYPGKF, respectively). In addition, the $\Delta 2-64$ ET_B receptor does not show any constitutive activity (see Fig. 17A) or increased cell surface expression in the presence of the antagonist BQ 788 (data not shown). Alternatively, the released N-terminal peptide could be involved in the cross-activation of another, yet unidentified receptor. Such a cross activation of PAR4 receptors by thrombin *via* PAR3 receptors has recently been described (Nakanishi-Matsui et al., 2000).

The ET_B receptor represents the first example of a type III membrane protein that is cleaved by a metalloprotease and, with exception of the alterations observed in ERK1/2 activation, retains its functional activity: ligand binding properties, G protein signaling and internalization. In contrast, in the case of the vasopressin V_2 receptor and the β_2 -adrenergic

receptor the structural integrity of the heptahelical receptor is destroyed, and in the case of the TSH receptor the ligand binding domain is lost.

4.6 Outlook

Since only the full-length ET_B receptor induced biphasic ERK1/2 activation, shedding of the N-terminal fragment could provide a key for switching from biphasic to monophasic ERK1/2 activation. Thus, limited proteolysis modulates the functional activity of the ET_B receptor. However, further studies are needed to identify the role of the biphasic ERK1/2 activation. As the intensity and the duration of ERK1/2 activation determine cellular response (e.g. proliferation or differentiation; (York et al., 1998; Zhang et al., 2000), the N-terminal proteolysis of the ET_B receptor could promote a phenotypic switching: from differentiation to proliferation. This finding could be of particular importance, as de-differentiation and increased metalloprotease activity play a significant role in numerous pathological processes, such as vascular and cardiac remodeling, tumor invasion, metastasis and atherosclerosis (Blobel, 2000; Garcia-Touchard et al., 2005). Since the agonist-dependent cleavage of the full-length ET_B receptor occurs at a site that is potentially targeted by serine proteases (SLAR/SLA), it could be possible that thrombin or plasmin cleave the ET_B receptor in an agonist-independent manner in the absence of ET-1. Interestingly, ET_B receptors are upregulated in VSMCs of atherosclerotic lesions, where they could play a preventive role promoting re-differentiation of de-differentiated cells (Dagassan et al., 1996; Iwasa et al., 1999). However, high levels of serine proteases in the vessel wall could change the ET_B receptor's effect in VSMCs from promoting re-differentiation to proliferation by an agonist-independent N-terminal shedding. Thus, regulation of protease activity could be of particular importance for the treatment of atherosclerosis, leading to a new pharmacological approach in the therapy of vascular disease.