# 4. Discussion

## 4.1 Homo- and Heterodimerisation of endothelin receptor subtypes.

Earlier investigations on the dimerisation of the endothelin receptors provided no direct evidence for the formation of homo- and heterodimers. Harada and co-workers reported that the $ET_B$ receptor in the anterior pituitary gland of rats does not independently bind ET-1 without the aid of the $ET_A$ receptor. From this observation the authors hypothesised that ET-1 may bridge the two receptors, which could result in the formation of an $ET_A$-$ET_B$ heterodimer (Harada et al. 2002). In another study chemical cross linking of $^{125}$ET-1 to sheep choroid plexus membranes revealed the existence of a number of bands with different molecular masses corresponding to the $ET_A$ and $ET_B$ receptors (Angelova et al. 1997). The authors speculated that ET-1 binding to the $ET_A$ receptor could result in the formation of receptor dimers. However, both studies lack a direct proof of dimerisation.

The current study shows by immunoprecipitation and FRET experiments with HEK293 cells co-expressing either $ET_A$myc.CFP and $ET_B$.YFP receptors or native $ET_A$ and $ET_B$ receptors the presence of $ET_A$myc.CFP/ $ET_B$.YFP and $ET_A$/$ET_B$ receptor heterodimers. Furthermore FRET experiments with HEK293 cells co-expressing $ET_A$myc.CFP and $ET_A$myc.YFP or $ET_B$.CFP and $ET_B$.YFP receptors demonstrated the existence of endothelin receptor homodimers. Since FRET efficiencies for $ET_A$myc.CFP/ $ET_B$.YFP dimers were decreased upon co-expression with native $ET_A$ and $ET_B$ receptors (without CFP/YFP moieties), it can be concluded that dimerisation is mediated by the receptor moieties. FRET measurements of cells expressing $ET_A$myc.CFP and $ET_B$.YFP receptors in the range between 0.5 and 5 pmol/mg of protein revealed that the level of dimerisation was independent of the expression level. Similar results were obtained in bioluminescence resonance energy transfer analysis with homo- and heterodimers of $\beta_1$- and $\beta_2$-adrenergic receptors, in which constant bioluminescence resonance energy transfer signals were found in the range of

1.4 to 26 pmol/mg of protein (Mercier et al. 2002). It is notable that a variety of cells that endogenously co-express $ET_A$ and $ET_B$ receptors show high levels of endothelin receptors comparable to those found in transfected HEK293 cells. For example, for epithelial cells of the choroid plexus, and astrocytes, $B_{max}$ values for $^{125}$I-ET1 were 0.12, 5.8 and 4.1 pmol/mg protein respectively (Angelova et al. 1997; Sasaki et al. 1998; Harada et al. 2002). The ratios of $ET_A$ and $ET_B$ receptor expression varied between 70:30 and 30:70 for epithelial cells of the choroid plexus and for astrocytes, respectively (Sasaki et al. 1998; Harada et al. 2002).

### 4.2 Regulation of $ET_A$/$ET_B$ dimerisation.

FRET experiments with living HEK293 cells co-expressing $ET_A$myc.CFP and $ET_B$.YFP receptors show that incubation with antagonists (BQ788, BQ123) or short-term incubation (5 min) with different agonists (ET-1, BQ3020) does not significantly influence the level of $ET_A$/$ET_B$ receptor heterodimers. In addition, the heterodimers remain stable upon prolonged incubation with the mixed agonist ET-1 (30 min). In a study by Himeno et al. the existence of $ET_A$ and $ET_B$ receptor heterodimers has been proposed on the basis of binding experiments in the anterior pituitary gland (Himeno et al. 1998). In this study it was shown that the $ET_B$ receptor could be detected only by radiolabelled endothelin-1 peptide binding in the presence of an $ET_A$-selective antagonist. The authors concluded that ET-1 is a bivalent ligand that has two distinct receptor recognition sites, one each for the $ET_A$ and $ET_B$ receptors. They proposed that ET-1 induced or stabilised the heterodimeric conformation. The present data demonstrate that $ET_A$ and $ET_B$ receptors form constitutive homo- and heterodimers and that ET-1 does not induce oligomerisation. Constitutive dimerisation has also been reported for other GPCRs like the rhodopsin receptor (Davies et al. 2001; Liang et al. 2003) and the dopamine $D_2$ receptor (Guo et al. 2003). Strikingly, prolonged incubation (30 min) with the $ET_B$ receptor-selective agonist BQ3020 resulted in the dissociation of $ET_A$/$ET_B$

receptor heterodimers. The dissociation was prevented either by addition of 450 mM sucrose or by co-expression of dominant negative K44A.dynamin I, which both inhibit clathrin-mediated internalisation (Damke et al. 1994; Oksche et al. 2000). The data strongly suggest that binding of BQ3020 to the heterodimer does not cause dissociation. Rather, the dissociation of the heterodimer takes place along the endocytic pathway. Several GPCRs have also been reported to undergo ligand-induced dissociation, including the δ-opioid receptor (Cvejic et al. 1997), the human cholecystokinin receptor type A (Cheng et al. 2001), the human thyrotropin receptor (Latif et al. 2002), the rhesus monkey neuropeptide Y4 receptor (Berglund et al. 2003) and the $hSST_2$ receptor (Grant et al. 2004). The $hSST_2$ receptor was found in FRET analysis to be a self-associated dimer at the cell surface in stably transfected cells. Ligand activation of $hSST_2$ receptors resulted in the dissociation of receptor dimers to monomers. Using a cell-impermeable cross-linking agent prevented agonist induced receptor dissociation. The authors concluded that dimer dissociation occurred on the plasma membrane. However, they also observed that in the presence of the cross-linking agent agonist induced receptor internalisation was impaired, which could in fact mean that the $hSST_2$ receptor dimer dissociation occurs, similarly to the endothelin receptor dimers, along the endocytic pathway.

### 4.3 Dimerisation of GPCRs. A general phenomena among all GPCR families?

Early studies using cross-linking, radioligand binding and radiation inactivation had predicted the existence of dimeric GPCRs (Salahpour et al. 2000). However the significance of these findings was not clear. More recent biochemical and biophysical studies have demonstrated that dimerisation occurs across all GPCR families (see Tab. 6 and 7). Most of those studies demonstrating the dimerisation of GPCRs, including the current work, used a combination of co-immunoprecipitation and resonance energy transfer experiments. However, strongest evidence that family A GPCRs exists as dimers derives from atomic force microscopy studies on the organisation of

rhodopsin and opsin in native disc membranes (Liang et al. 2003). Rhodopsin and opsin form structural dimers that are organised in paracrystaline arrays. The intradimer links might be provided by contacts between transmembran helices IV and V, with additional organisational structure derived from interactions involving transmembrane helices I and II.

Family B receptors form heterodimers with a family of chaperone proteins, called RAMPs (receptor activity modifying proteins). The calcitonin receptor-like receptor (CRLR) dimerises with one of three different RAMPs, which assist in the transport of the receptor to the cell surface. Moreover RAMPs define the glycosylation state and the affinity for different ligands of the receptor heterodimer (McLatchie et al. 1998).

Family C GPCRs including the $GABA_B$ receptors (Jones et al. 1998; Kaupmann et al. 1998; White et al. 1998), the calcium-sensing receptor (Bai et al. 1998; Jensen et al. 2002), the metabotropic glutamate receptor (Romano et al. 1996) and the sweet taste receptors (Nelson et al. 2001; Li et al. 2002; Nelson et al. 2002), have been shown to assemble as preformed dimers. Here dimerisation is important for proper targeting of the receptors to the cell surface and for G protein activation (see also 4.4 functional role of GPCR dimerisation)

Tab. 7: **Heterodimerisation of GPCRs.**

| Receptors | Reference |
|---|---|
| **Heterodimerisation between subtypes** | |
| $GABA_{B1}$ and $GABA_{B2}$ | (Jones et al. 1998; Kaupmann et al. 1998; White et al. 1998) |
| T1R2 and T1R3 | (Nelson et al. 2001) |
| T1R1 and T1R3 | (Nelson et al. 2002) |
| $D_2$ and $D_3$ dopamine | (Scarselli et al. 2001) |
| $M_2$ and $M_3$ muscarinic | (Maggio et al. 1999) |
| δ- and κ-Opioid | (Jordan et al. 1999) |

| | |
|---|---|
| δ- and μ-Opioid | (George et al. 2000; Gomes et al. 2000) |
| $SST_1$ and $SST_5$ | (Rocheville et al. 2000) |
| $SST_{2A}$ and $SST_3$ | (Pfeiffer et al. 2001) |
| $SST_2$ and $SST_{3a}$ | (Pfeiffer et al. 2001) |
| 5-$HT_{1B}$ and 5-$HT_{1D}$ | (Xie et al. 1999) |
| β1-adrenergic and β2-adrenergic | (Lavoie et al. 2002; Mercier et al. 2002) |
| $MT_1$ and $MT_2$ | (Ayoub et al. 2002) |
| $TRH_1$ and $TRH_2$ | (Hanyaloglu et al. 2002) |
| $CCR_2$ and $CCR_5$ | (Mellado et al. 1999; Mellado et al. 2001) |
| $CCR_2$ (V64I) and $CCR_5$ | (Mellado et al. 1999) |
| $CCR_2$ (V64I) and $CXCR_4$ | (Mellado et al. 1999) |
| S1P1 and S1P2 | (Van Brocklyn et al. 2002) |
| S1P1 and S1P3 | (Van Brocklyn et al. 2002) |
| S1P2 and S1P3 | (Van Brocklyn et al. 2002) |
| **Heterodimerisation between different receptors** | |
| $SST_{2A}$ and μ-Opioid | (Pfeiffer et al. 2002) |
| $AT_1$ angiotensin and $B_2$ bradykinin | (AbdAlla et al. 2000; AbdAlla et al. 2001) |
| $SST_5$ and $D_2$ dopamine | (Rocheville et al. 2000) |
| δ-Opioid and β2-adrenergic | (Jordan et al. 2001; McVey et al. 2001; Ramsay et al. 2002) |
| κ-Opioid and β2-adrenergic | (Jordan et al. 2001; Ramsay et al. 2002) |
| $A_{2A}$ adenosine and mGluR5 | (Ferre et al. 2002) |
| $A_{2A}$ adenosine and $D_2$ dopamine | (Hillion et al. 2002) |
| $A_1$ adenosine and $D_1$ dopamine | (Gines et al. 2000) |
| $A_1$ adenosine and $P2Y_1$ purinergic | (Yoshioka et al. 2001; Yoshioka et al. 2002) |
| mGlu and Ca | (Gama et al. 2001) |

Tab. 8: **Homodimerisation of GPCRs.**

| Receptors | Reference |
|---|---|
| β1-adrenergic | (Mercier et al. 2002) |
| β2-adrenergic | (Hebert et al. 1996; Hebert et al. 1998; Angers et al. 2000; Angers et al. 2001; Mercier et al. 2002) |
| $D_2$ dopamine | (Ng GY 1996; Zawarynski et al. 1998; Lee et al. 2000) |
| $D_3$ domamine | (Nimchinsky et al. 1997; Elmhurst et al. 2000; Karpa et al. 2000) |
| δ-Opioid | (Cvejic and Devi 1997; Jordan and Devi 1999; McVey et al. 2001; Ramsay et al. 2002) |
| κ-Opioid | (Jordan and Devi 1999; Ramsay et al. 2002) |
| $H_2$ histamine | (Fukushima et al. 1997) |
| $M_3$ muscrinic | (Zeng et al. 1999) |
| 5-$HT_{1B}$, 5-$HT_{1D}$ | (Xie et al. 1999) |
| $MT_1R$, $MT_2R$ | (Ayoub et al. 2002) |
| $CCR_2$ | (Rodriguez-Frade et al. 1999) |
| $CCR_5$ | (Benkirane et al. 1997; Vila-Coro et al. 2000; Blanpain et al. 2002; Issafras et al. 2002) |
| $CXCR_4$ | (Vila-Coro et al. 1999) |
| $SST_1$, $SST_4$, $SST_5$ | (Rocheville et al. 2000) |
| $B_2$ bradykinin | (AbdAlla et al. 1999) |
| Oxytocin | (Terrillon et al. 2003) |
| $V_{1a}$ vasopressin | (Terrillon et al. 2003) |
| $V_2$ vasopressin | (Zhu et al. 1998; Terrillon et al. 2003) |
| TRH | (Kroeger et al. 2001) |
| Angiotensin | (Monnot et al. 1996) |
| MSH | (Carrithers et al. 1996) |

| | |
|---|---|
| Bombesin | (Carrithers and Lerner 1996) |
| LH | (Indrapichate et al. 1992; Roess et al. 2000) |
| IgG-Hepta | (Abe et al. 1999) |
| GnRH | (Conn et al. 1982; Cornea et al. 2001; Horvat et al. 2001; Kroeger et al. 2001; Cornea et al. 2002) |
| $mGlu_{1a}$ | (Robbins et al. 1999; Kunishima et al. 2000; Tsuji et al. 2000) |
| $mGlu_5$ | (Romano et al. 1996) |
| CaR | (Bai et al. 1998; Bai et al. 1999; Jensen et al. 2002) |
| alpha-Mating factor | (Overton et al. 2000) |
| $S_1P_1$, $S_1P_2$, $S_1P_3$ | (Van Brocklyn et al. 2002) |

### 4.4 Functional role of GPCR dimerisation.

*Ontogeny:* GPCR dimerisation has been implicated in modulating receptor folding and transport to the cell surface. Detailed analysis of the metabotropic γ-aminobutyric acid ($GABA_B$) receptor showed that co-expression of the $GABA_{B1}$ and the $GABA_{B2}$ receptors was required for the formation of a functional $GABA_B$ receptor at the cell surface (Kaupmann et al. 1998; White et al. 1998; Kuner et al. 1999). When expressed alone $GABA_{B1}$ is retained intracellularly as an immature protein because it has a carboxy-terminal ER retention motiv (Couve et al. 1998). By contrast, $GABA_{B2}$ reaches the cell surface, even if expressed alone, but is not functional (White et al. 1998). Heterodimerisation between the two receptor subtypes masks the $GABA_{B1}$ ER retention signal, allowing the proper transport of the functional $GABA_{B1/B2}$ receptor heterodimers to the plasma membrane (Margeta-Mitrovic et al. 2000). Whether $ET_A/ET_B$ receptor heterodimerisation occurs initially during biosynthesis is not clear. In the current study mainly complex glycosylated heterodimers could be detected. Most mature forms of family A GPCRs become terminally *N*-

glycosylated, which is probably a key quality control step before export from the Golgi apparatus. If this requires a protein-protein interaction provided by dimerisation, similar to the family C $GABA_B$ receptors, is not fully understood.

*Pharmacological diversity:* Heterodimerisation of $ET_A$ and $ET_B$ receptors does not alter the affinities of $ET_A$ or $ET_B$ receptors to ET-1, ET-3, and BQ123 in HEK293 cell clones. Moreover the endothelin receptor subtypes displayed similar affinities to ligands when expressed individually or in combination. However hetero-oligomerisation can result in receptor complexes that display different ligand binding properties. For κ- and δ-opioid receptor dimers no significant affinity for either κ- or δ-opioid receptor-selective agonists or antagonists have been observed. In contrast the heterodimer showed high affinity for partially selective ligands (Jordan and Devi 1999). In addition, these selective ligands were found to bind the heterodimer synergistically when added simultaneously.

*Internalisation:* The heterodimerisation of endothelin receptor subtypes results in a delayed sequestration of the $ET_B$ receptor, when stimulated with ET-1. Similar observations were made for heterodimers of δ/κ-opioid, $\beta_2$-adrenergic/κ-opioid, $\beta_1/\beta_2$-adrenergic and cholecystokinin $CCK_A/CCK_B$ receptors (Jordan and Devi 1999; Jordan et al. 2001; Lavoie et al. 2002; Cheng et al. 2003). Apparently, the slower internalising receptor subtype determines the rate of internalisation in the heterodimer. In the case of the $ET_A/ET_B$ receptor heterodimer, the retarded internalisation is overcome by stimulation with an $ET_B$ receptor-selective agonist. The mechanisms causing dissociation of $ET_A/ET_B$ receptor heterodimers upon treatment with BQ3020 remains elusive. It is possible that the different ligands induce the exposure of different endocytic motifs. In the ET-1-bound heterodimer, transport signals may be exposed which promote recycling to the cell surface, similar to the trafficking of the $ET_A$ receptor when expressed individually (Bremnes et al. 2000). In contrast, BQ3020 may expose transport motifs, which direct the $ET_B$ receptor into late endosomal/lysosomal compartments, similar as the $ET_B$ receptor when expressed alone (Bremnes et al. 2000; Oksche et al. 2000). During intracellular

trafficking the $ET_A$ receptor may dissociate from the $ET_B$ receptor, e.g. in the acidic environment of endosomes and recycle to the plasma membrane. The mechanism by which homo- or heterodimers interact with a single adapter protein, which directs endocytosis or intracellular trafficking, remains speculative. Molecular modelling of complexes consisting of the rhodopsin homodimer and either visual arrestin or transducin (based on the available crystal structures of the three proteins) suggest that the rhodopsin homodimer interacts with a single visual arrestin or transducin molecule (Liang et al. 2003). According to this model, a homo and/or heterodimer could only recruit one adaptor protein, e.g. arrestin. This would prevent the interaction with another, second adaptor protein simply for sterical reasons. It is possible that the BQ3020-stimulated heterodimer binds a different adaptor protein than the ET1-stimulated heterodimer.

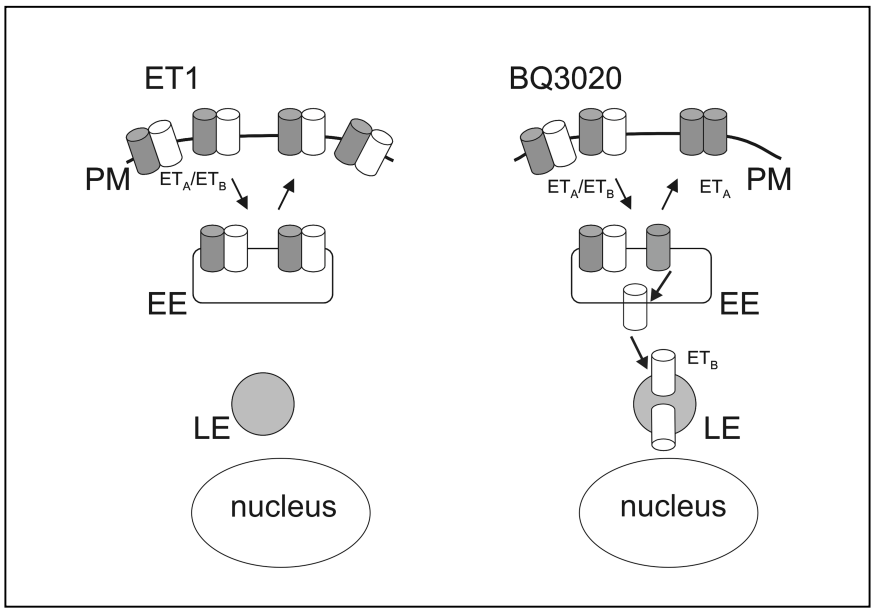


Fig. 37. **Modell of the $ET_A$/$ET_B$ heterodimer dissociation.** ET-1 induces the internalisation of $ET_A$/$ET_B$ heterodimers and promotes recycling of both receptor subtypes to the cell surface. In contrast, BQ3020 mediates the dissociation of the heterodimers along the endocytic pathway. The $ET_A$ receptor recycles to the cell surface whereas the $ET_B$ receptor is targeted to late endosomal compartments. PM, plasma membrane; EE, early endosome; LE, late endosom.

## 4.5 Outlook, enhancing and disrupting GPCR oligomerisation.

Heterodimerisation in particular altered levels of heterodimers have been linked to clinical disorders. In pre-eclamtic hypertensive women it was found that the level of angiotensin $AT_1$/bradykinin $B_2$ heterodimers is significantly increased (AbdAlla et al. 2001). Furthermore, evidence indicated that hypertension in pre-eclamsia is related to the increase of $AT_1/B_2$ receptor heterodimerisation. This example demonstrates that in some cases GPCR heterodimerisation might represent the molecular basis of human disease. As noted earlier, agonists might regulate GPCR receptor dimerisation. Therefore drugs, which specifically disrupt or enhance GPCR dimers might also regulate dimerisation-dependent functions. A specific drug induced dissociation of $AT_1/B_2$ heterodimers, similarly to the BQ3020 mediated dissociation of $ET_A/ET_B$ heterodimers, could result in reduced levels of $AT_1/B_2$ heterodimers and thus might lead to normotensive blood pressure in pre-eclamtic hypertensive women.

The present results show that $ET_A$ and $ET_B$ receptors form constitutive heterodimers. However, heterodimerization is reversible. The dissociation of the heterodimer is specifically mediated by an $ET_B$-selective agonist and depends on endocytosis. Moreover, dissociation of the endothelin receptor dimers is important for efficient receptor trafficking, since heterodimerisation alters ET-1 stimulated $ET_B$ receptor internalisation. Because the $ET_B$ receptor is crucially involved in the clearance of ET-1 from the circulation (Saito et al. 1991; Dupuis et al. 1996), ET-1-mediated internalisation of the $ET_B$ receptor may have functional consequences. The switch of the intracellular trafficking mode of the $ET_B$ receptor from down-regulation to recycling in the heterodimer changes not only the number of functional $ET_B$ receptors on the cell surface, but also the clearance of ET-1. The physiological role of $ET_A/ET_B$ receptor dimers remains to be shown. Since $ET_A$ and $ET_B$ receptors are co-expressed in atherosclerotic vessels, it is valuable to prove whether heterodimerisation alters signalling

properties of $ET_A$ and $ET_B$ receptors to enhance vasoconstriction, proliferation or differentiation.