Endothelin A and Endothelin B Receptors Differ in Their Ability to Stimulate ERK1/2 Activation

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Endothelin-1 (ET-1) acts on two different G protein–coupled receptors, namely the endothelin A (ET_A) and the endothelin B (ET_B) receptors. Both receptor subtypes show differences in their tissue expression and signal transduction. In the present study, we compared the ability of ET_A and ET_B receptors to stimulate extracellular signal-regulated kinase 1/2 (ERK1/2). In addition, we analyzed the role of the extracellular N terminus for ERK1/2 activation, because the ET_B receptor undergoes an agonist-dependent N-terminal proteolysis. ET-1 stimulation of HEK293 cells stably expressing the ET_A receptor induced a monophasic, but sustained ERK1/2 activation, whereas the ET_B receptor showed a biphasic ERK1/2 activation. A truncated mutant ET_B receptor, lacking the proteolytically cleaved N terminus (Δ2-64 ET_B) revealed only a monophasic and transient ERK1/2 activation. Treatment of HEK293 Δ2-64 ET_B cell clones with ET-1 and a synthetic NT27-64 peptide, corresponding to the N-terminally cleaved fragment of the ET_B receptor and ET-1, did not restore the biphasic activation of ERK1/2. A chimeric ET_B receptor in which the N terminus was replaced by the N terminus of the ET_A receptor elicited biphasic ERK1/2 activation. The presented data suggest that an intact N terminus of the ET_B receptor is necessary for the second phase of ERK1/2 activation. However, it appears that the length of the N terminus rather than a specific sequence motif is required for biphasic ERK1/2 activation. Exp Biol Med 231:757–760, 2006

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Introduction

Endothelins (ET-1, ET-2, and ET-3) are vasoactive peptides (1), which exert their action *via* two G protein–coupled receptors: the endothelin A (ET_A) and the endothelin B (ET_B) receptor (2, 3). The ET_A receptor is mainly expressed in vascular smooth muscle cells, and its stimulation induces a long-lasting vasoconstriction (4). The ET_B receptor is predominantly expressed in endothelial cells, and its activation causes a transient vasodilation (5). Although both endothelin receptors share 64% amino acid sequence identity, marked differences in the activation of G proteins exist. Whereas the ET_A receptor activates G proteins of the $G_{q/11}$ and $G_{12/13}$, the ET_B receptor stimulates G proteins of the G_i and $G_{q/11}$ families (6–8). In addition, the ET_B receptor displays several unique features that are not shared by the ET_A receptor. For example, only the ET_B receptor undergoes an agonist-dependent proteolysis of its N terminus. Although both endothelin receptor subtypes possess a cleavable signal peptide (ET_A receptor: 16 aa, ET_B receptor: 26 aa) that is removed by a signal peptidase in the endoplasmic reticulum lumen during receptor biosynthesis (9–11), only the ET_B receptor reveals a further proteolytic cleavage within the extracellular N terminus (10). This cleavage occurs 38 amino acids C-terminally of the signal peptidase cleavage site, between arginine 64 and serine 65 (R64/S65) and is mediated by a metalloprotease in an agonist-dependent manner (12). Because the proteolytically released N-terminal peptide fragment harbors the only N-linked glycosylation site (N59/A60/S61), agonist-mediated proteolysis results in a truncated, nonglycosylated receptor. However, the physiologic significance of this process remains elusive.

Both, ET_A and ET_B receptors lead to an activation of extracellular signal-regulated kinase 1/2 (ERK1/2). Because ERK1/2 is important regulator of cellular proliferation, migration, and differentiation, we analyzed whether ET_A and ET_B receptors differ in their ability to activate ERK1/2.

In addition, we studied whether N-terminal proteolysis of the ET_B receptor is involved in ERK1/2 activation.

Materials and Methods

Peptide Synthesis. Synthesis of ET-1 and of the NT27-64 peptide (corresponds to the N-terminal fragment of the ET_B receptor released by a metalloprotease) were carried out by the solid-phase, standard 9-fluorenylmethoxycarbonyl chemistry method as described recently (13).

Generation of ET_B Receptor/GFP Expression Constructs. The plasmids pcDNA3.ETB, pEGFP.ETB, and pΔ2-64.ETB encoding the human full-length ET_B receptor, the human ET_B/GFP fusion protein, and a mutant ET_B receptor with a truncated extracellular N terminus (corresponding to the N-terminally cleaved ET_B receptor), respectively, have been described previously (12, 13). Plasmids encoding a chimeric ET_A receptor in which the N terminus is replaced by the N terminus of the ET_B receptor (ANB·GFP), and a chimeric ET_B receptor in which the N terminus is replaced by that of the ET_A receptor (BNA·GFP) were generated by site-directed mutagenesis of the pEGFP.ETB and pEGFP.ETA plasmids (13). A *Sna*BI (underlined) site in the coding region of both plasmids was introduced using the following primers: for the ET_B receptor: 5′-GAGA CTTT CAAA TACG TAAA CACT GTGA TA- 3′; reverse: 5′-TATC ACGT GTTT ACGT ATTT GAAA GTCTC- 3′; for the ET_A receptor: 5′-TCAG CTTT CAAA TACG TAAA CACG GTTG TG- 3′; reverse: 5′- CACA ACCG TGTT TACG TACG TATT TGAA AGCT GA- 3′. The resulting plasmids pETA·GFP.*Sna*BI and pETB·GFP.*Sna*BI were then subjected to endonuclease treatment with *Sna*BI. Because within the promoter region of pETA·GFP.*Sna*BI and pETB·GFP.*Sna*BI, a second *Sna*BI site is present, the *Sna*BI digest results in fragments of 560 base pairs and 5454 base pairs (ET_A receptor) and 634 base pairs and 5433 base pairs (ET_B receptor). Subsequently, the 560 base-pairs fragment of pETA·GFP.*Sna* BI was ligated with the 5433 base-pairs fragment of pETB·GFP.*Sna*BI, and the 5454 base-pairs fragment of pETA·GFP.*Sna*BI was ligated with the 634 base-pairs fragment of pETB·GFP.*Sna*BI. The sequences of the resulting plasmids pBNA·GFP and pANB·GFP were verified by DNA sequencing using the Big Dye Terminator kit (Applied Biosystems, Weiterstadt, Germany).

Cell Culture and Transient Transfections. HEK293 cell clones stably expressing the ET_B·GFP, BNA·GFP, ANB·GFP, or the Δ2-64 ET_B·GFP receptor were maintained as described (12). Growth arrest was induced by culturing cells for 48 hrs in a serum-free quiescent medium. The protocol for the transient transfection of HEK293 cells was as described previously (14).

Immunoblots. Detection of total ERK1/2 and phospho-ERK1/2 in immunoblots was performed as described (12, 15). In brief, HEK293 cells were grown on 35-mm Petri dishes, stimulated with ET-1 (100 n*M*) for up to 3 hrs and finally lysed in Laemmli buffer. Proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gels, blotted to nitrocellulose filters (Schleicher und Schuell, Dassel, Germany) and probed with affinity-purified polyclonal phospho-ERK1/2 (detects phosphoERK1/2) or ERK1/2 antibodies (detects phosphorylated and non-phosphorylated forms of ERK1/2) (both 1:1000) (Cell Signaling Technology, Danvers, MA).

Results and Discussion

To analyze whether ET_A and ET_B receptors differ in their ability to activate ERK1/2, we studied HEK293 cells stably expressing the full-length ET_B·GFP receptor or the ET_A·GFP receptor. HEK293 cells were chosen for these experiments because nontransfected cells lack endogenous expression of endothelin receptors (no specific binding of ^{125}I-ET-1 and no ET-1–induced ERK1/2 activation; data not shown). Cell clones expressing ET_A·GFP or ET_B·GFP receptors were stimulated with ET-1 for up to 3 hrs, then lysed, and samples were subjected to immunoblot analysis using phosphoERK1/2 and ERK1/2 antibodies. Lysates derived from HEK293 cells expressing ET_A·GFP showed a monophasic, but sustained increase in ERK1/2 phosphorylation (Fig. 1A). For lysates derived from cells expressing the ET_B·GFP receptor, a biphasic ERK1/2 activation was found (Fig. 1B). The early phase of ERK1/2 activation reached a peak at 3–5 min and slowly declined thereafter within 30–60 min. The second phase was observed after 80 min of agonist application and persisted for up to 180 min (Fig. 1B). To study the role of the N-terminal proteolysis on ERK1/2 activation of the ET_B receptor, we also analyzed ERK1/2 phosphorylation in HEK293 cells stably expressing the Δ2-64 ET_B·GFP receptor. ET-1 stimulation of Δ2-64 ET_B·GFP did not show biphasic, but only monophasic ERK1/2 activation (Fig. 1C).

Because only the full-length ET_B receptor induced biphasic ERK1/2 activation, the presence of the N-terminal fragment could provide a key for switching from biphasic to monophasic ERK1/2 activation. To elucidate, whether the proteolytically released N-terminal peptide functions as a soluble ligand or coactivator in ET-1–induced ERK1/2 activation, HEK293 cell clones expressing the Δ2-64 ET_B·GFP receptor were incubated with a synthetic peptide (NT27-64, 100 μ*M*; Fig. 2A). This peptide corresponds to the proteolytically cleaved N-terminal fragment of the ET_B receptor, with the exception that it lacks an N-linked glycosylation at asparagine 59. Treatment of HEK Δ2-64 ET_B·GFP cell clones with the synthetic NT27-64 peptide and ET-1 did not restore the biphasic activation of ERK1/2 (Fig. 2A).

To study whether the length of the ET_B receptor or a certain amino acid composition within the ET_B receptor's N terminus is required for biphasic ERK1/2 activation, we analyzed a chimeric ET_B receptor in which the extracellular N terminus was replaced by that of the ET_A receptor

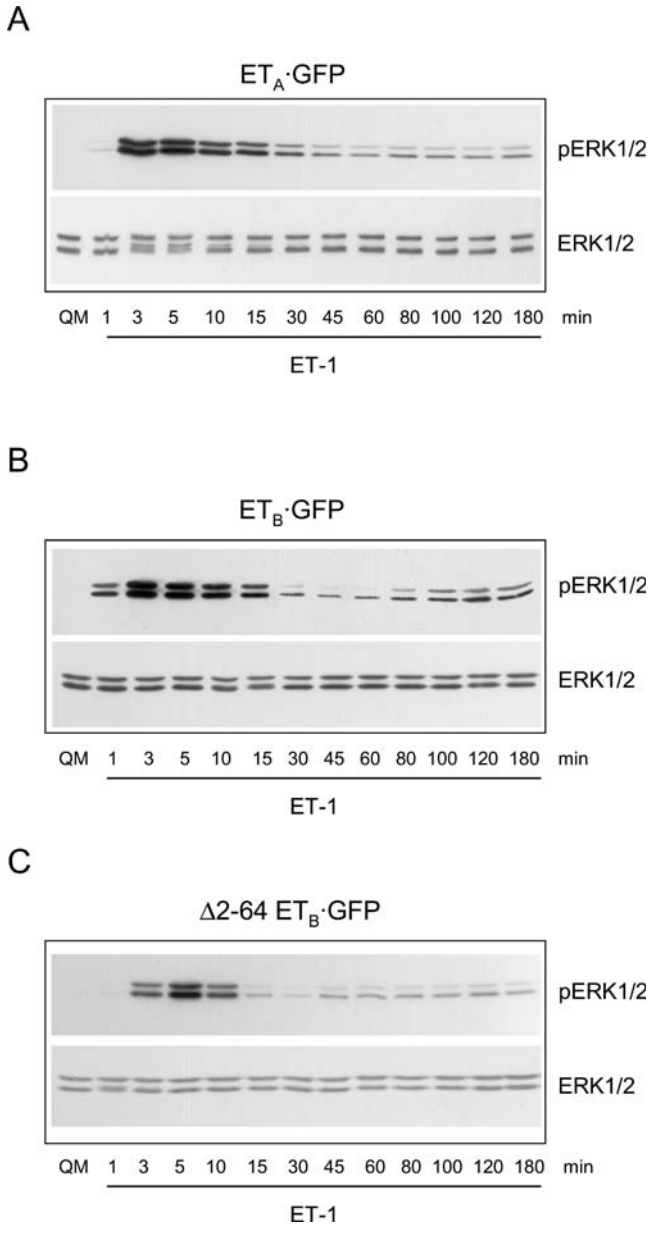

Figure 1. ET_A and ET_B receptors show differences in ET-1–mediated ERK1/2 activation. HEK293 cell clones expressing either ET_A·GFP (A) the full-length ET_B·GFP (B), or the N-terminally truncated ET_B·GFP receptor (Δ2-64 ET_B·GFP; C) were stimulated with 100 n*M* ET-1 for up to 180 min and finally lysed. Proteins were then separated by 10% sodium dodecyl sulfate-polyacrylamide gels, transferred to filters, and then probed with antiphosphoERK1/2 and anti-ERK1/2 antibodies. Shown are representative results of at least three independent experiments.

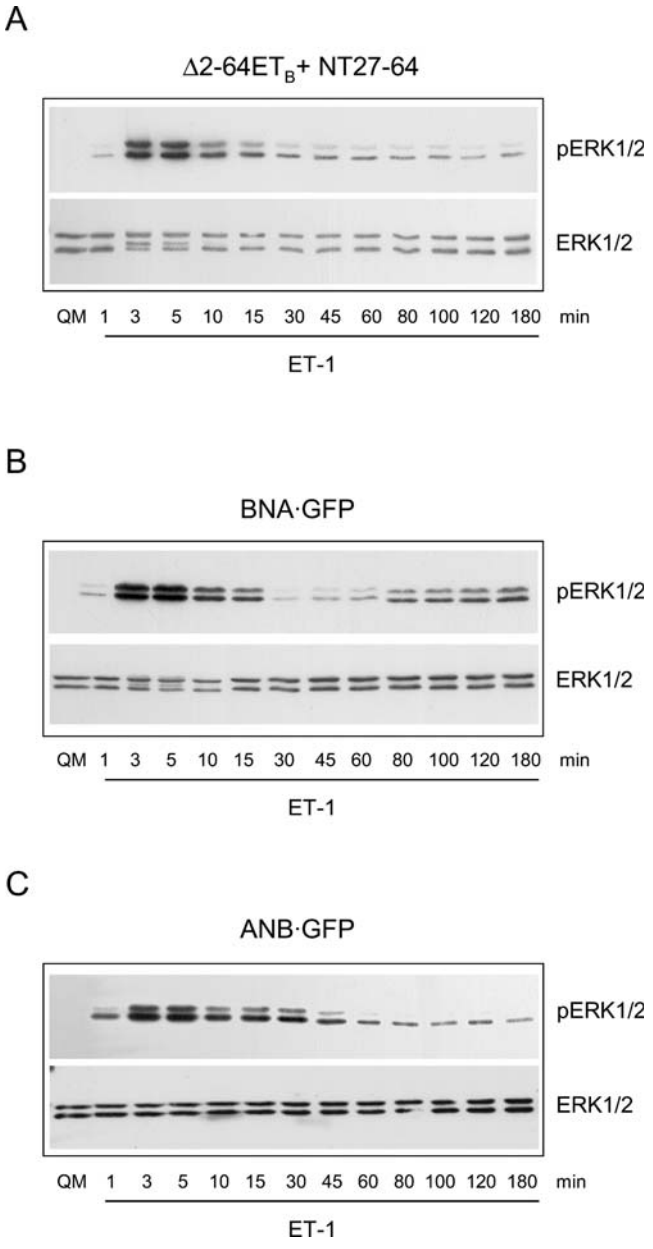

Figure 2. The second phase of ERK1/2 activation requires the presence of glycosylated endothelin receptors N terminus. HEK293 cell clones expressing the N-terminally truncated mutant Δ2-64 ET_B·GFP (A) were incubated with a synthetic, nonglycosylated N-terminal peptide (NT27-64, 100 μ*M*) for 30 mins before the stimulation of cells with ET-1 for up to 180 mins. HEK293 cells stably expressing a chimeric ET_B receptor, in which the N-terminus was replaced by that of the ET_A receptor (BNA·GFP) (B) or a chimeric ET_A receptor, in which the N terminus was replaced by that of the ET_B receptor (ANB·GFP) (C) were stimulated with 100 n*M* ET-1 for the indicated times. Lysates of cells were processed for immunoblotting as described in the legend to Figure 1.

(BNA·GFP) and also a chimeric ET_A receptor in which the N terminus was replaced by that of the ET_B receptor (ANB·GFP). The BNA·GFP chimera induced a biphasic ERK1/2 phosphorylation on ET-1 stimulation (Fig. 2B), similar to that seen for the wild-type ET_B receptor, and the ANB·GFP chimera elicited a monophasic, but sustained ERK1/2 activation as observed for the wild-type ET_A receptor (Fig. 2C). The data suggest that the intact, extracellular N terminus of the ET_B receptor is involved in biphasic ERK1/2 activation. Curiously, replacement of the extracellular N terminus of the ET_B receptor by the N terminus of the ET_A receptor did not alter biphasic ERK1/2 activation. This is notable because the extracellular N termini of the ET_A and ET_B receptors display only a little amino acid sequence identity.

The data demonstrate that ET_A and ET_B receptors differ considerably in their ability to stimulate ERK1/2 activation. Whereas the ET_A receptor shows a monophasic, but sustained ERK1/2 activation, the ET_B receptor elicits a biphasic ERK1/2 activation. Although an intact N terminus of the ET_B receptor is necessary for biphasic ERK1/2 activation, the extracellular ET_B receptor's N terminus itself is not sufficient for the second phase of ERK1/2 activation, because it can be replaced by the N terminus of the ET_A receptor. Regarding the low level of amino acid identity between the N termini of the ET_A and the ET_B receptor, it appears that the length of the N terminus rather than a specific sequence motif is required for biphasic ERK1/2 activation. In future experiments, the mechanisms involved in biphasic ERK1/2 activation of the full-length ET_B receptor have to be studied in more detail. In addition, further studies are planned to demonstrate whether differences in the pattern of ERK1/2 activation between ET_A and ET_B receptors also result in different cellular responses, such as proliferation and differentiation. This is highlighted by studies on the pheochromocytoma cell line PC12: whereas treatment of these cells with epidermal growth factor resulted in monophasic ERK1/2 activation and proliferation, incubation of cells with nerve growth factor elicited biphasic ERK1/2 activation and differentiation (16).

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