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PURIFICATION OF AN ENDOTHELIN RECEPTOR FROM HUMAN PLACENTA

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Summary: We have identified an endothelin (ET) binding protein on the membranes of human placenta and purified it to homogeneity. It is a polypeptide with an apparent Mol. Wt. of 40,000 and is a major protein to be labeled by cross-linking with either ^{125}I-ET-1, -2, or -3. Binding studies with Scatchard analysis indicated the presence of a single class, high-affinity binding site with Kds of 57 pM, 480 pM and 40 nM for ^{125}I-labeled ET-1, ET-2 and ET-3, respectively. These results suggest that the 40K protein is a major ET receptor in placenta and, most likely, can bind differentially to ET-1, ET-2 and ET-3.

Introduction: Endothelins are a group of strong vasoactive peptides recently isolated from the culture medium of endothelial cells (1). The vasoconstrictor actions of endothelin have been shown to increase the intracellular calcium concentration (2). Several lines of studies suggested that endothelins do not act directly on the previously known calcium channel (3), but rather on the specific receptor (4). Indeed, specific binding sites for ET-1 have been detected in various tissues (5-10). Also, proteins of diverse sizes (Mol. Wt. 32,000-128,000) were specifically labeled by cross-linking with ^{125}I-ET-1 suggesting the presence of multiple species of ET receptor (8, 11, 12).

To understand how endothelins regulate the intracellular calcium concentration without any direct involvement of the calcium channel, it is essential to isolate and characterize the receptor. In the present study we identified an endothelin binding protein in human placenta membranes, solubilized and purified it to homogeneity by affinity column chromatography.

Materials and Methods

Materials: ET-1, ET-2, and ET-3 were purchased from Peptide Institute Inc. (Osaka, Japan), and the ^{125}I-labeled isomers (specific activity: ET-1, 62 TBq/mmol; ET-2, 65 TBq/mmol; ET-3, 72 TBq/mmol) were from Amersham. Human placentas from Japanese women were supplied by local hospitals. 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), Disuccinimidyl suberate (DSS), and sulfosuccinimidyl 2-(biotinamido) ethyl-1,3-dithiopropionate (NHS-SS-biotin) were

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from Pierce Chemical Co. Prestained and regular molecular markers used for determination of the molecular weight of proteins by SDS-PAGE were from Bio-Rad.

Solubilization and partial purification of ET receptor from human placenta membrane: Human placentas were minced and homogenized in a Waring blender in two volumes of 50 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl, 5mM $MgCl_2$, 15 μg/ml each of chymostatin, antipain, pepstatin, leupeptin, 0.1 mg/ml soybean trypsin inhibitor and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 12,000 X g for 30 min. The pellet was suspended in two volumes of washing buffer and centrifuged. After multiple cycles of washing, the pellets were suspended in the solubilization buffer that contained 50 mM sodium phosphate buffer (pH 7.4), 1% CHAPS, 0.6 M NaCl and a mixture of the six protease inhibitors described above. The ET receptor was solubilized by stirring the mixture at 4°C. After an overnight extraction, the mixture was centrifuged at 21,000 x g for 30 min and the supernatant fraction (referred to as crude extract), which contained over 90% of ET-1 binding activity, was obtained. The crude extract was fractionated with 4-20% (w/v) polyethyleneglycol 6,000 (PEG). The ET receptor, precipitated with 20% PEG, was stable for more than two weeks when stored at 4°C. For further purification, the pellet was dissolved in 25 mM sodium phosphate buffer (pH 7.4) and fractionated stepwise, by precipitation with lowering the pH. A major ET-1 binding activity was present in the supernatant fraction of pH 5.5 and it was neutralized to pH 7.0. The neutralized fraction (2.0 g protein in a total 100ml) was then loaded onto a Phenyl Sepharose column (6.5 cm x 7 cm), washed with 25 mM sodium phosphate buffer (pH 7.0)-10% glycerol, and eluted with 0.1 M glycine-NaOH buffer (pH 10.5)-1% CHAPS-10% glycerol. The ET binding activity was concentrated and dialyzed against 50 mM sodium phosphate buffer (pH 7.4)-10% glycerol-0.1% CHAPS. For extensive purification, the crude ET receptor was subjected to affinity column chromatography that used biotinylated ET-1 derivative and immobilized avidin agarose (Pierce). In brief, the partially purified ET receptor (Phenyl Sepharose fraction, 30 ml), in 50 mM sodium phosphate buffer (pH 7.4)-0.1% CHAPS-10% glycerol was incubated at 25°C with biotinylated ET-1 which was prepared similarly as described in reference 13. After a 90 min incubation, the sample was loaded onto an avidin agarose column (5 ml). The column was washed successively with 50 mM sodium phophate buffer (pH 7.4)-0.2 M NaCl and, 10 mM sodium phosphate buffer (pH 7.4)-0.1% CHAPS, and eluted with 10 mM sodium phosphate buffer (pH 7.4)-0.2M β-mercaptoethanol. The ET receptor-ET-1 derivative complex was released from the column after dissociation of ET-1 and biotinyl residues by reducing the disulfide linkage between them. The principle of this affinity chromatography has been previously described (13). The eluate was dialyzed, concentrated and analyzed by 11% polyacrylamide gel electrophoresis in the presence of 0.1 % sodium dodecyl sulfate (SDS-PAGE) as shown in Fig. 4 (14).

Binding assay: The assay mixture (0.05 ml), consisting of 50 mM sodium phosphate buffer (pH 7.4), 0.1% CHAPS, 1 mg/ml bovine serum albumin and ET receptor (1-10 μg of protein) was incubated with 62.5 pM ^{125}I-ET (1 x 10^4 cpm) for 30 min at room temperature (25°C). Nonspecific binding was determined in the presence of 500 nM nonradioactive ET-1. The receptor-^{125}I-ET complex was separated from free ^{125}I-ET by mixing 0.05 ml of 15%(v/v) calcium phosphate gel which precipitated the complex. The pellet was washed with 0.3 ml of 50 mM sodium phosphate buffer (pH 7.4)-0.2% CHAPS and was isolated by centrifugation (15,000 rpm, for 1 min) and the radioactivity was counted with a γ-counter.

Results

Crosslinking of ET-1 to membrane-associated and solubilized receptor. A polypeptide of apparent molecular weight 40,000 was specifically and strongly labeled when human placenta membranes were incubated with ^{125}I-ET-1 and crosslinked with DSS (Fig. 1. lanes 1 and 2). The ET binding protein solubilized from membranes was subjected to similar crosslinking conditions as used for membranes. The lanes 3 and 4 of Fig. 1 clearly showed that a protein of same Mol. Wt. was also labeled, indicating that human placenta contained a major 40K endothelin receptor. Similar specific labeling profiles were also obtained with ^{125}I-ET-2 as well as ^{125}I-ET-3 (data not shown).

Purification of 40K endothelin receptor. Results in the initial stages of purification were summarized in Table 1. Several experiments were carried out with the fraction obtained by Phenyl Sepharose column chromatography to test the stability as well as the ET binding activities. The binding studies indicated that the solubilized receptor has Kd values 57 pM, 480 pM and 40 nM for ET-1, ET-2 and ET-3, respectively (Fig. 2, Panel A, B and C). The ^{125}I-ET-1 binding was inhibited by ET-1 and ET-2, at the Ki values of 0.5 nM and

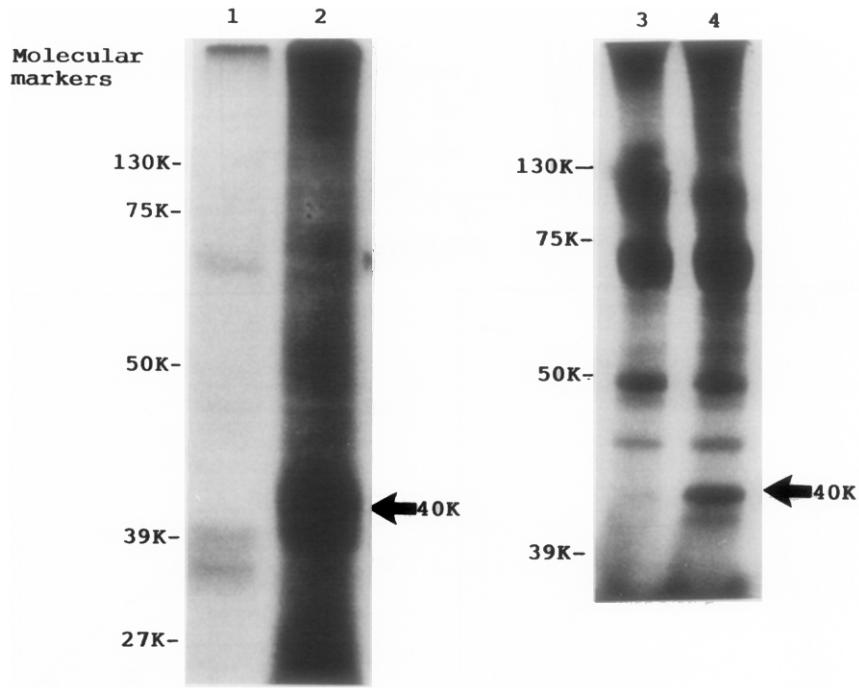

Fig. 1: Affinity labeling of ET receptors by crosslinking to ^{125}I-ET-1. ET receptors in membranes and the solubilized extracts were crosslinked by DSS as described previously by Watanabe et al. (8). The crosslinked receptor was analyzed by 11% SDS-PAGE after it was boiled in 60 mM Tris-HCl buffer (pH 6.8) containing 2% SDS and 5% β-mercaptoethanol.
Lane 1: membranes plus 10 mM ET-1. Lane 2: membranes. Lane 3: solubilized fraction plus 10 mM ET-1. Lane 4: solubilized fraction.

Table 1. Purification of receptor from human placenta

Fractions	Total activity pmol ET	Total protein mg	Specific ET binding pmol ET/mg
Crude extract	240	3,600	0.066
PEG precipitation	294	2,760	0.107
Acid treatment	267	2,090	0.128
Phenyl Sepharose	195	830	0.235

1.5 nM. ET-3 failed to inhibit the activity at this range of concentration. The 50% inhibition by ET-3 occurs at a much higher concentration (0.4 μM). The 40K ET receptor has a wide pH range for the binding activity as showin in Fig.

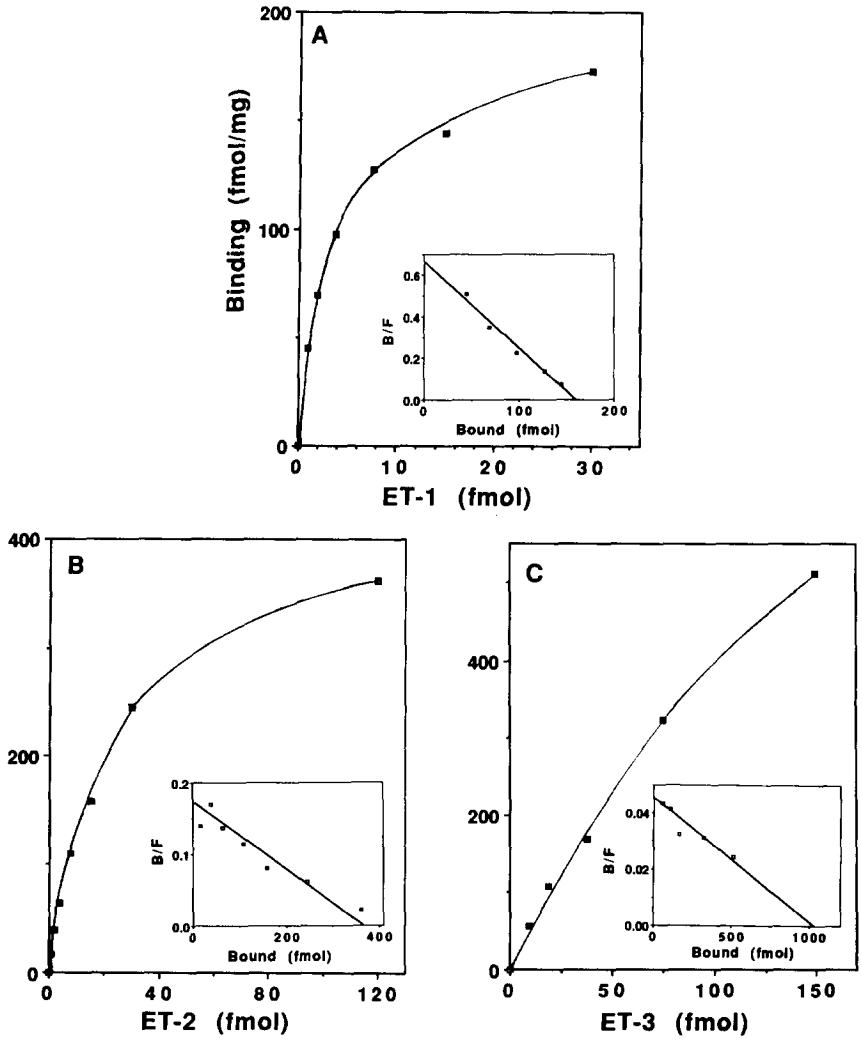

Fig. 2: Binding of human placenta ET receptor to three endothelin subtypes. Partially purified receptor fraction (a preparation after Phenyl Sepharose column chromatography) was incubated with ^{125}I-labeled ET-1, -2 or -3. Insets represent the Scatchard plot analyses.

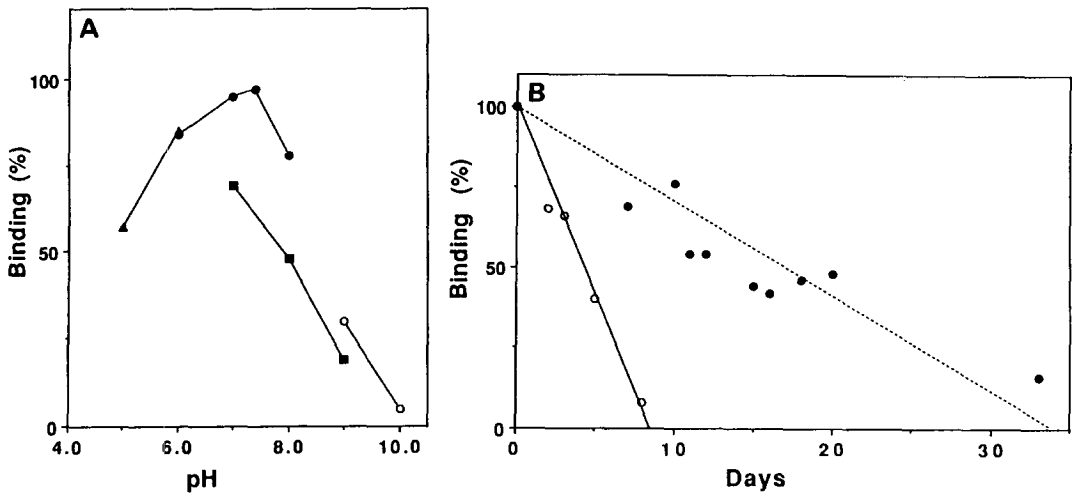

Fig. 3: Stability of partially purified ET receptor.
Panel A: The ET binding activity was determined under various pH conditions. Buffers used in the experiments were 100 mM sodium acetate buffer (▲—▲), 100 mM sodium phosphate buffer (●—●), 100 mM Tris-HCl buffer (■—■), and 100 mM glycine-NaOH buffer (○—○).
Panel B: Stability of the ET-binding activity of receptor was determined for two different preparations, i.e., the receptor fraction after acid treatment (●---●) and the partially purified receptor fraction after Phenyl Sepharose column chromatography (○——○) (Table 1). Samples were stored at 4°C as indicated in 50 mM sodium phosphate buffer (pH 7.4) containing 0.1% CHAPS and 10% glycerol.

3, A. At pH 7.4, the receptor showed a short half-life of 4 days at 4 °C (Fig. 3, B). Because of these observations we predicted that there would be difficulty in proceeding with a purification scheme limited to conventional column chromatography.

Purification of endothelin receptor by affinity column chromatography. To reduce the time and steps required for the purification, we adopted an affinity column chromatorgraphy that used avidin agarose and the preformed complex of endothelin receptor and biotinylated-ET-1 derivative. Procedures for affinity chromatography were described in the Materials and Methods. To ensure the isolation of endothelin-specific receptor, a portion of sample protein was incubated with 0.5 μM ET-1 before mixing with biotinylated ET-1. As shown in Fig. 4-B, Lane 3, preincubation with ET-1 did reduce the formation of receptor-biotinylated ET-1 complex and the recovery of receptor from avidin column. Analysis of eluted protein by SDS-PAGE (Fig. 4, B) clearly demonstrated that the endothelin receptor specifically reacted with the biotinylated ET-1 derivative, bound to avidin agarose and eluted by reduction with ß-mercaptoethanol. The Mol. Wt. of the protein thus obtained was indeed 40,000 daltons (Fig. 4, A), which is consistent with the previous data obtained by crosslinking studies (Fig. 1).

Discussion

Endothelins exert a strong and long-acting vasoconstrictor activity on smooth muscle cells (1, 2, 7). These observations imply that the endothelins

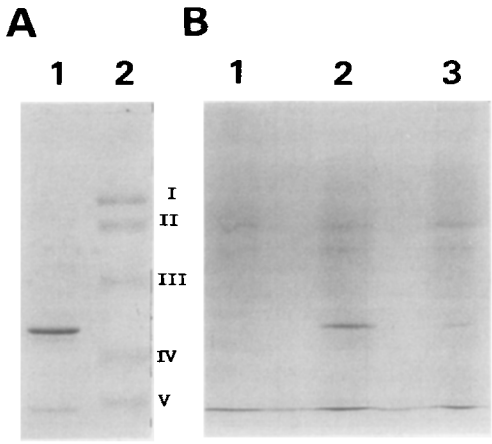

Fig. 4: Isolation of human placenta ET receptor by affinity column chromatography. Characterization of the protein eluted from avidin agarose column was carried out by 11% SDS-PAGE, after boiling the samples in the presence of 2% SDS-5% β-mercaptoethanol.
Panel A: Lane 1, purified ET receptor (stained with Coomassie Brilliant Blue); lane 2, Bio-Rad prestained molecular markers (I, 130K phophorylase b; II, 75K bovine serum albumin; III, 50K ovalbumin; IV, 34K carbonic anhydrase (corrected from the original 39K); V, 27K soybean trypsin inhibitor.
Panel B: Affinity chromatography specific for ET receptor purification. Lane 1, no sample; lane 2, no preincubation with ET-1 prior to the ET receptor-biotinyl ET-1 complex formation; lane 3, preincubated with ET-1 before complex formation. (note that the intensity of purified ET receptor band was diminished specifically upon preincubation with ET-1). Proteins were stained by silver.

are a family of peptides which play the important role in the regulation of systematic blood pressure and local blood flow. To investigate ultimately the molecular mechanisms for these important roles, we purified the endothelin receptor present in human placenta membrane. The major receptor protein is a polypeptide of 40,000 daltons. There was another, a smaller polypeptide with a Mol. Wt. of 38,000 daltons that appeared as a minor component (Fig. 4, A).

During our present studies, Nakajo et al. reported the presence of one type of ET-1 receptor on human placental membranes, and its solubilization (12). From crosslinking studies with ^{125}I-ET-1, they estimated a Mol. Wt. of 32,000 for the human placenta receptor which contradicts with our present results. Further studies remains to be directed to clear this particular discrepancy, however, we feel that the 40K endothelin receptor may contain sensitive proteolytic sites which produce either 32K or 38K polypeptides from a 40K receptor.

An important observation obtained from the present study is that all three endothelin subtypes could bind to the 40K polypeptide(s). It is likely that a single receptor species, rather than multiple species of receptors of identical molecular weight, reacts in human placenta with different endothelins and has

different binding constants. The less selective ET receptor of this type has been reported previously for the rat caudate putamen (15).

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