Vol. 164, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1989 Pages 205-211

SOLUBILIZATION AND IDENTIFICATION OF HUMAN PLACENTAL ENDOTHELIN RECEPTOR

Shigeo Nakajo, Masanori Sugiura, Rudolf M. Snajdar, Frank H. Boehm and Tadashi Inagami

Departments of Biochemistry and Gynecology and Obstetrics, Vanderbilt University, School of Medicine, Nashville, TN 37232

Received August 29, 1989

SUMMARY. Endothelin-1 (ET-1) receptor was identified on the membranes from human placenta and 66% of original binding activity in the membranes was solubilized with 0.75% (w/v) CHAPS. Binding studies of the solubilized membranes using ^{125}I-ET-1 indicated the presence of a single class of high-affinity binding sites with an apparent Kd of 760 pM and a Bmax of 1.8 pmol/mg of protein. The binding was inhibited by addition of unlabeled ET-1 and ET-3 in dose dependent manner. The Ki values of solubilized membranes were 84 pM for ET-1 and 250 pM for ET-3, whereas particulate membranes had weaker affinities (Ki=410 pM for ET-1, 2500 pM for ET-3). Calcium channel blockers such as nicardipine, verapamil and diltiazem did not affect the binding of ^{125}I-ET-1. Affinity labeling of the particulate and solubilized membranes with CHAPS revealed a specific binding protein with a Mr of 32,000.

Endothelins are a group of potent vasoconstrictor peptides recently isolated from culture medium of endothelial cells (1). The vasoconstrictor actions of ET have been shown to be due to increase in intracellular calcium (2). It appears, however, that endothelin does not act directly on the calcium channel but rather on a separate and specific receptor.

Specific binding sites for ET-1 have been detected in diverse tissues, including various cultured vascular cells (3-6), and other types of cells and tissues (7-18). Affinity labeled binding sites were identified in chick cardiac membranes (19) rat renal glomeruli (20), and rat mesangial cells (21). Proteins of various sizes (34,000-128,000) were specifically labeled suggesting heterogeneity in ET receptors. However, no report has been published on further purification and characterization of the endothelin binding site.

To clarify the signal transduction mechanism it is paramount to isolate and characterize the endothelin receptor. In the present study, we examined the binding of ^{125}I-ET-1 to human placental membranes, determined the conditions

The abbreviations used are: CHAPS, 3-[(3-cholamidopropyl) dimethylammonio)]-1-propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazinelthanesulfonic acid; EGTA: [ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid; SDS, sodium dodecyl sulfate.

0006-291X/89 $1.50

for the solubilization of the ET-1 receptor from these membranes, and using chemical cross-linking, identified the human ET-1 binding protein.

MATERIALS AND METHODS

Materials. ET-1 was radioiodinated with [^{125}I] Na (New England Nuclear) using chloramine T (22). The monoiodinated ET-1 was purified by HPLC with gradient elution of 20-80% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid from Vydac C18 column. The specific activity of ^{125}I-ET-1 was approximately 2,000 Ci/mmol.

Purified Triton X-100, CHAPS and bis(sulfosuccinimidyl) suberate (BS^3) were purchased from Pierce Chemical Co. Leupeptin, aprotinin, PMSF, bacitracin, pepstatin, marker proteins for gel electrophoresis, octyl-β-D-glucoside, diltiazem, verapamil and nicardipine were from Sigma. Sodium deoxycholate, sodium cholate, $C_{12}E_8$ and zwittergent(3-12) were from Calbiochem. Angiotensin II, atrial natriuretic factor (1-28) and [Arg^8]-vasopressin were from Peninsula Laboratories, Inc. ET-1 was obtained from Peptides International, Inc. All other reagents were of analytical grade.

Preparation of Particulate Fraction from Human Placenta. All procedures were performed at 4°C unless otherwise specified. Human placentas were washed with phosphate-buffered saline, then minced and homogenized in 4 volumes of Buffer A consisting of 20 mM HEPES, pH 7.4, 5 mM EDTA, 3 mM EGTA, 0.4 mM PMSF, 3 μg/ml leupeptin, 2 μg/ml aprotinin, 0.25 mg/ml bacitracin, and 3 μg/ml pepstatin containing 0.25 M sucrose. Placentas were homogenized in a Waring blender for two 20 sec bursts followed by 20 sec Polytron homogenization. The homogenate was then centrifuged at 8,000 x g for 20 min. The supernatant was further centrifuged at 105,000 x g for 60 min and the resultant precipitate was resuspended in an equal volume of Buffer A containing 0.15 M NaCl. The suspension was homogenized in a Dounce homogenizer then centrifuged at 105,000 x g for 60 min. The precipitate thus obtained was suspended in buffer A containing 0.15 M NaCl followed by a dispersion with a Dounce homogenizer. This particulate fraction was stable for at least three months when kept at -80°C .

Binding Assay. Binding assay mixture (0.2 ml) consisting of 30 mM HEPES, pH 7.5, 0.15 M NaCl, 5 mM $MgCl_2$, 0.5 mg/ml bacitracin, 0.4 mM PMSF (Buffer B), 1 mg/ml BSA and 35-80 μg of membrane protein was incubated with 75 pM ^{125}I-ET-1 for 2 hr at 4°C. Nonspecific binding was determined in the presence of 125 nM nonradioactive ET-1. The receptor-^{125}I-ET-1 complex was separated from free ^{125}I-ET-1 by filtration through Whatman GF/B glass fiber filters which were prewetted with 0.3% polyethyleneimine as described previously (23).

Cross-Linking of ET-1 Receptor. Particulate fraction (175 μg of protein) and solubilized membranes (120 μg of protein) were incubated in 0.1 ml of Buffer B for 2 hr at 4°C with 300 pM ^{125}I-ET-1 in the presence or absence of 0.125 μM unlabeled ET-1, 1 μM angiotensin II or 1 μM [Arg^8]-vasopressin. After incubation, 2 μl of 40 mM BS^3 was added, and incubated for 25 min at 4°C. The reaction was terminated by the addition of a 5-fold concentrated sample buffer (0.313 M Tris-HCl, pH 6.8, 10% (w/v) SDS and 40% (v/v) glycerol) in the presence or absence of 25% (v/v) β-mercaptoethanol and heated for 3 min in a boiling water bath. The samples were immediately electrophoresed on a 10% polyacrylamide gel by the method of Laemmli (24). For autoradiography, the gels were dried, placed on Kodak XAR-5 films with Dupont Cronex Lightening-Plus intensifying screens, and exposed for 2-5 days at -80°C.

Solubilization of ET-1 receptor. Frozen membrane preparations (2.9 mg protein/0.25 ml) were thawed at 4°C and an equal volume of various detergent solutions were added to make final concentrations as indicated. These

suspensions were stirred for 90 min at 4°C and subsequently centrifuged at 105,000 x g for 60 min. The binding activity in each supernatant (30 μl) was measured as described above.

RESULTS

Endothelin-1 bound specifically to the membranes prepared from human placenta. The binding reached an apparent equilibrium after 1 hr incubation at 22°C or 2 hr incubation at 4°C. The dissociation rate of ^{125}I-ET-1 from ligand-receptor complex was extremely slow; approximately 85% of the maximum binding was still observed at 24 hr after the addition of 1,600-fold excess unlabeled ET-1.

In order to clarify further the molecular properties of the ET-1 receptor, we attempted to solubilize the ET-1 receptor from human placental membranes by using various detergents (Table I), used at concentrations higher than their respective critical micellar concentration. CHAPS was the most effective detergent which solubilized 66% of original ET-1 binding activity. In contrast, $C_{12}E_8$ and octyl-β-D-glucoside were ineffective.

The binding of ^{125}I-ET-1 to particulate and solubilized preparations was saturable (Fig. 1A) and the Scatchard analyses (Fig. 1B) indicated a single class of high affinity binding sites for these preparations. The particulate and CHAPS

Table I

SOLUBILIZATION OF ET-RECEPTOR WITH VARIOUS DETERGENTS FROM PARTICULATE FRACTION OF HUMAN PLACENTA

Detergent	Concentration (%)	Specific Binding[a] (cpm)	Recovery of [b] Binding Activity (%)
Triton X-100	0.5	10032 ± 417	57
$C_{12}E_8$	0.015	N.D.	--
CHAPS	0.75	11678 ± 332	66
Sodium deoxycholate	0.4	11361 ± 427	64
Sodium cholate	0.9	9672 ± 681	55
Zwittergent (3-12)	0.2	3069 ± 139	17
Octyl-β-D-glucoside	0.8	310 ± 190	2
None	--	N.D.	--

[a] Data are mean ± S.D. [b] Each value represents a percentage of the specific binding activity recovered in the solubilized fraction in reference to the maximum binding to the particulate membranes.

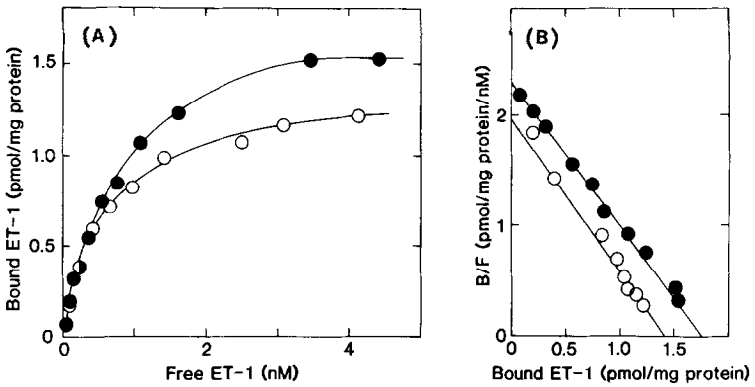

Fig. 1. Saturable binding of ^{125}I-ET-1 to the particulate and solubilized membranes (A) and the Scatchard analyses (B). The particulate membranes (○) (58 μg of protein) and the solubilized membranes (●) (44 μg of protein) were incubated with increasing concentrations of ^{125}I-ET-1 at 4°C for 2 hr. Nonspecific binding was determined by parallel incubations in the presence of 125 nM nonradioactive ET-1 and was 5-15% of the total binding.

solubilized membranes had Bmax values of 1.4 and 1.8 pmol/mg of protein, respectively, and Kd values of 700 and 760 pM, respectively. The apparent Kd value for membranes did not change significantly after solubilization.

Competition binding analyses were performed using varying concentration of unlabeled ET-1, ET-3 and Ca^{2+} channel blockers against a fixed concentration of ^{125}I-ET-1 (Fig. 2). Unlabeled ET-1 and ET-3 inhibited specific binding in a concentration-dependent manner. The calculated Ki values of ET-1 and ET-3 for particulate membranes were 410 pM and 2,500 pM respectively, whereas for

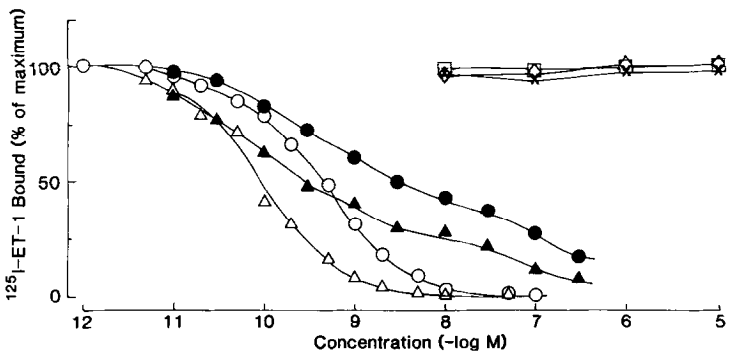

Fig. 2. Competition between ^{125}I-ET-1 and increasing concentrations of ET-1, ET-3 and calcium channel blockers. The particulate membranes (●, ○) (58 μg of protein) and the solubilized membranes (▲, △) (44 μg of protein) were incubated with fixed concentrations of ^{125}I-ET-1 (75 pM) and the designated concentrations of ET-1 (○, △) and ET-3 (●, ▲) at 4°C for 2 hr. Nicardipine (x), verapamil (◇) and diltiazem (□) were also incubated with the particulate membranes and fixed concentration of ^{125}I-ET-1 as described above. Binding data were corrected for nonspecific binding which was determined as described for Fig. 1 and expressed as percentages of the maximal ^{125}I-ET-1 binding.

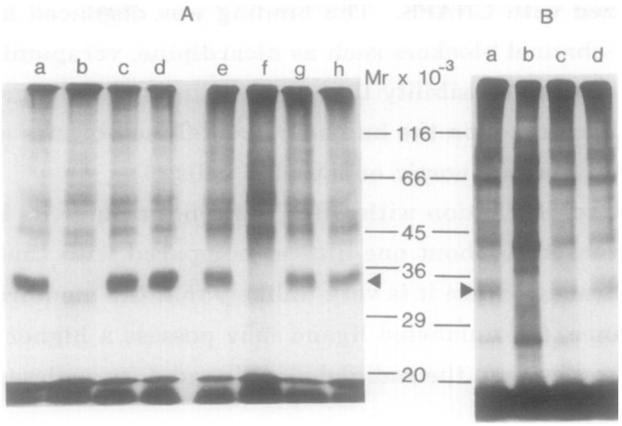

Fig. 3. Autoradiograms of the particulate and solubilized membranes which were cross-linked with ^{125}I-ET-1. The particulate and the solubilized membranes were cross-linked with ^{125}I-ET-1 using BS^3 as described under "Materials and Methods", and electrophoresed under non-reducing (A) and reducing (B) conditions. Chemical cross-linking experiments were carried out in the absence (lanes a and e) or presence (lanes b and f) of 0.125 μM unlabeled ET-1, 1 μM angiotensin II (lanes c and g) and 1 μM [Arg^8]-vasopressin (d and h). Molecular weight standards used were β-galactosidase (116,000), BSA (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000) and trypsin inhibitor (20,100). Arrow heads indicate the position of specifically labeled ET-1 binding protein.

solubilized membranes were 84 pM and 250 pM, respectively. The affinities of ET-1 for the particulate or the solubilized membranes were 3 - 6-fold higher than for those of ET-3. In contrast, neither nicardipine, verapamil nor diltiazem, up to 10^{-5} M, affected the binding of ^{125}I-ET-1.

Chemical cross-linking experiments were carried out with ^{125}I-ET-1. As shown in Fig. 3A, one predominant band with a molecular weight of 32,000 (indicated by arrow head) was labeled. This band was displaced in both the particulate and CHAPS solubilized membranes by excess unlabeled ET-1 as visualized on a 10% gel without β-mercaptoethanol (compare lane a with lane b and lane e with lane f in Fig. 3A). Although intensity of the 32,000 protein band in the solubilized membranes decreased under the reducing condition (Fig. 3B lane A), the protein was also quenched by excess cold ET-1 (compare lane a with lane b in Fig. 3B). On the other hand, the binding of ^{125}I-ET-1 to 32,000 protein was not affected either by angiotensin II, [Arg^8]-vasopressin (lanes c,d,g and h in Fig. 3A, and lanes c and d in Fig. 3B) or atrial natriuretic factor (result not shown).

DISCUSSION

In the present study, we demonstrated that human placental membranes have a high-affinity specific binding site for ET-1 and that ET-1 binding activity

could be solubilized with CHAPS. The binding was displaced by ET-1 and ET-3 but not by Ca^{2+}-channel blockers such as nicardipine, verapamil and diltiazem. This result excludes the possibility that endothelin is an endogenous agonist of the L-type calcium channel on the human placental membranes and differs from results obtained in porcine aortic endothelial cells (1).

Though the solubilization with CHAPS did not change its binding affinity, the Ki value decreased to about one-fifth as compared with that obtained from particulate membranes. Since it is very unlikely that the membranes contain ET or ET-like substance, the unlabeled ligand may possess a higher affinity for the solubilized membranes than the radiolabeled ligand does as described previously (20).

From the saturation studies, the human placental membranes appear to have a single binding domain for ET-1 (Fig. 1). A few reports suggested that there were multiple receptor subtypes for ET-1 on chick cardiac membranes (19), rat renal glomeruli (20) and rat mesangial cell membranes (21). When the human placental membranes solubilized with CHAPS was cross-linked with ^{125}I-ET-1 using BS^3, only one predominant band with a molecular weight of 32,000 was specifically labeled as visualized on a 10% polyacrylamide gel in the presence or absence of β-mercaptoethanol. Similarly sodium deoxycholate solubilized membranes also gave a specifically labeled band of 32,000 molecular weight (date not shown). Taken together these results indicate that one type of ET-1 receptor exists on human placental membranes and that disulfide bonds do not contribute to the subunit structure.

ACKNOWLEDGMENTS

We wish to thank Ms. Doris Bruey for typing the manuscript. This work was supported by research grants HL 35323 and HL 14192 from the National Institutes of Health.

REFERENCES

1. Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mistsui, Y., Yazaki, Y., Goto, IK. and Masaki, T. (1988) Nature 332, 411-415.
2. Auguet, M., Delaflotte, S., Chabrier, P.E., Pirotzky, E., Clostre, F. and Braquet, P. (1988) Biochem. Biophys. Res. Commun. 156, 186-192.
3. Hirata, Y., Yoshimi, H., Takata, S., Watanabe, T.S., Kumagai, S., Nakajima, K. and Sakakibara, S. (1988) Biochem. Biophys. Res. Commun . 154, 868-875.
4. Marsden, P.A., Danthuluri, N.R., Brenner, B.M., Ballermann, B.J. and Brock, T.A. (1989) Biochem. Biophys. Res. Commun. 158, 86-93.
5. Chabrier, P.E., Roubert, P., Longchampt, M.O., Gillard, V., Plas, P., Goulin, J. and Braquet, P. (1989) The FASEB J. 3, A719.
6. Clozel, M., Fischli, W. and Guilly, C. (1989) J. Clin. Invest. 83, 1758-1761.
7. Hirata, Y., Fukuda, Y., Yoshimi, H., Emori, T., Shichiri, M. and Marumo, F. (1989) Biochem. Biophys. Res. Commun. 160, 1438-1444.
8. Kohzuki, M., Johnston, C.I., Chai, S.Y., Casley, D.J. and Mendelsohn, F.A.O. (1989) Eur. J. Pharmacol. 160, 193-194.

9. Simonson, M.S., Wann, S., Meme, P., Dubyak, G.R., Kester, M., Nakazato, Y., Sedor, J.R. and Dunn, M.J. (1989) J. Clin, Invest. 83, 708-712.
10. Badr, K.F., Munger, K.A., Sugiura, M., Snajdar, R.M., Schwartzberg, M. and Inagami, T. (1989) Biochem. Biophys. Res. Commun. 161, 776-781.
11. Martin, E.R., Brenner, B.M. and Ballermann, B.J. (1989) Kidney Int., 35, 316 (Suppl. 1).
12. Orita, Y., Fujiward, Y., Ochi, S., Takama, T., Fukunaga, M. and Yokoyama, K. (1989) J. Cardiovascular Pharmacol. (Suppl. 5), S159-S161.
13. Gu, X.H., Casley, D.J. and Nayler, W.G. (1989) J. Cardiovascular Pharmacol. (Suppl. 5), S171-S173.
14. Cozza, E.N., Vila, M.C., Gomez-Sanchez, C.E., Chiou, S. and Foecking, M.F. (1988) Clin. Res. 37, 11A.
15. Cozza, E.N. and Gomez-Sanchez, C.E. (1989) Clin. Res. 37, 552A.
16. Power, R.F., Wharteon, J., Salas, S.P., Kanse, S., Ghatei, M., Bloom, S.R. and Polak, J.M. (1989) Eur. J. Pharmacol. 160, 199-200.
17. Miyazaki, H., Kondoh, M., Watanabe, H., Hayashi, T., Murakami, K., Takahashi, M., Yanagisawa, M., Kimura, S., Goto, K. and Masaki, M. (1989) J. Cardiovascular Pharmacol. (Suppl. 5), S155-S156.
18. Fischli, W., Clozel, M. and Guilly, C. (1989) Life Sci. 44, 1429-1436.
19. Watanabe, H., Miyazaki, H., Kondoh, M., Masuda, Y., Kimura, S., Yanagisawa, M., Masaki, T. and Murakami, K. (1989) Biochem. Biophys. Res. Commun. 161, 1252-1259.
20. Martin, E.R., Marsden, P.A., Brenner, B.M. and Ballermann, B.J. (1989) Biochem. Biophys. Res. Commun. 162, 130-137.
21. Sugiura, M., Snajdar, R.M., Schwartzberg, M., Badr, K.F. and Inagami, T. (1989) Biochem. Biophys. Res. Commun. (in press).
22. Hunter, W.M. and Greenwood, F.C. (1962) Nature 194, 495-496.
23. Takayanagi, R., Inagami, T., Snajdar, R.M., Imada, T., Tamura, M. and Misono, K. (1987) J. Biol. Chem. 262, 12104-12113.
24. Laemmli, U.K. (1970) Nature 227, 680-685.