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DETECTION OF AN ENDOTHELIN-1-BINDING PROTEIN COMPLEX BY LOW TEMPERATURE SDS-PAGE

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We found that the complex of ET-1 and its binding protein was stable enough to be separated by SDS-PAGE when electrophoresis was run at a low temperature. Cross-linking was not necessary for the detection of [^{125}I]-ET-1 and its binding protein complex by autoradiography.

This simple method could be used in qualitative (estimation of apparent molecular weight of ET-1 binding protein) and quantitative (determination of relative content of ET-binding protein) analysis of the ET-binding protein complex. ET-binding protein complexes of various animal species and organs were investigated by this method.

Endothelin-1 has been reported to be a strong vasoconstrictor secreted by vascular endothelial cells (1). Evidence of its various biological functions in a variety of organs and cells has been described (2-7). The specific binding of ET-1 to membranes or solubilized fractions from various organs and cultured cells has also been reported.

The binding proteins of ET-1 have been mainly examined with a cross-linking method and SDS-PAGE (8-14). In general, the protein-protein or peptide-protein interaction is broken during

Abbreviations

SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; CHAPS,3-{(3-cholamidopropyl) dimethylammonio} -1-propanesulfonate; BSA, bovine serum albumin; ET, endothelin; BPB, bromophenol blue.

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SDS-PAGE. Covalent cross-linking is, therefore, necessary for the detection of such protein (peptide)-protein complex.

The complex of ET-1 and its binding protein is very stable and the release of ET-1 from cells or membranes prelabeled with ET-1 is very slow (15-17). The complex is also stable at pH 6-8 (18, 19), or in the presence of 0.5 % CHAPS. These findings prompted us to study the comigration of ET-1 and its binding protein in SDS-PAGE.

The results indicated that ET-1-binding protein complexes are stable enough to survive as a complex in SDS-PAGE if it is run at a low temperature. We found that the low temperature SDS-PAGE is a powerful tool for the clarification of the nature of ET binding protein of various sources.

Materials and Methods

Materials: ET-1 was purchased from Peptide Institute Inc. (Osaka, Japan) and the [^{125}I]-labeled ET-1 (81.4 TBq/m mol) was from New England Nuclear. CHAPS was from Dojindo Laboratories. Prestained molecular markers used for SDS-PAGE was from Bio-Rad.

Preparation of CHAPS extract: Adult beagle dogs and guinea pigs were anethesized with pentobarbital, killed by exsanguination and then the organs were removed. Mice were killed by cervical dislocation. Human placenta was obtained from local hospitals. 2-5 g of each type of organ tissue was minced and homogenized with a Polytron in 10 volumes of homogenizing buffer containing 0.25 M sucrose, 10 mM sodium phosphate (pH 7.4), 1 mM EDTA and protease inhibitors (5 μg/ml of pepstatin, leupeptin, chymostatin, trypsin inhibitor and antipine). The homogenate was centrifuged at 2,500 x g for 10 min at 4 °C. The supernatant was collected and centrifuged at 100,000 x g for 1 h at 4 °C. The 100,000 x g pellet was suspended in an equal volume of the homogenizing buffer and stored at -130 °C (microsomal fraction). An aliquot of the microsomal fraction was mixed with an equal volume of extraction buffer containing 20 mM sodium phosphate pH 7.4, 1 % CHAPS, 1M NaCl and protease inhibitors. After 1 h of extraction on ice, the sample was centrifuged at 100,000 x g for 1 h at 4 °C and the supernatant (referred to here as the CHAPS extract) was obtained.

Detection of the complex of [^{125}I]-ET-1 and its binding protein on SDS-PAGE without cross-linking

The CHAPS extract (containing 10-20 μg protein) and [^{125}I]-ET-1 (final concentrations of 0.01-1.5 nM, 500-75,000 cpm) was

mixed and incubated for 1 h at room temperature. The sample was cooled on ice and equal volume of sample buffer containing 2 % SDS, 5 % β-mercaptoethanol, 20 % glycerol, 0.01 % BPB and 0.25 M Tris-HCl (pH 6.8) was added. Electrophoresis was performed in a 10% polyacrylamide gel in the presence of 0.1 % SDS at a constant current of 40 mA. The gel plate was kept cool by circulating ice cold water through a tube submerged in the buffer tank during electrophoresis. The gels were dried between two sheets of cellophane paper at 85 °C for 30 min and exposed to Kodak X-Omat AR film with two sheets of intensifying screen at -80 °C for 3-24 h.

Calcium phosphate gel binding assay

The CHAPS extract (10-20 μg protein) and [^{125}I]-ET-1 (final concentrations of 0.01-1.5 nM, 500-75,000 cpm) were mixed and incubated for 1h at room temperature. Fifty microliters of 15 % (v/v) calcium phosphate gel suspension and 0.5 ml of washing buffer containing 50 mM sodium phosphate buffer (pH 7.4), 0.2 % CHAPS was added to the reaction mixture. After a brief centrifugation (4,500 x g, 10 sec) the supernatant was removed. The pellet was resuspended in 0.5 ml of the buffer and centrifuged. The radioactivity in washed pellet was counted by a gamma counter. Nonspecific binding was determined in the presence of 1 μM nonradioactive ET-1.

Results

Detection of stable complex of endothelin and its binding protein after low temperature SDS-PAGE: When the mixture containing the CHAPS extract from the microsomal fraction and [^{125}I]-ET-1 was incubated for 1 h at room temperature, and then SDS-PAGE was performed without cross-linking, clear discrete bands of [^{125}I]-ET-1 and its binding protein complex were observed after autoradiography. Fig. 1 shows the results obtained with the canine cerebellum microsomal fraction. The bands at the front of the gel revealed unbound [^{125}I]-ET-1. The ET-1-binding protein complexes, which migrated at apparent molecular weights of 55 kD and 35 kD, were clearly detected. The intensity of the radioactive band increased as the concentration of [^{125}I]-ET-1 was raised, and reached its maximum at about 700 pM of [^{125}I]-ET-1 (Fig. 1A, B). The saturation pattern was consistent with the data obtained with the calcium phosphate gel binding assay (18). The summation of the radioactivity of the 55 kD and 35 kD bands was about the half of that obtained with the calcium phosphate gel binding assay. This may be due to the partial release of [^{125}I]-ET-

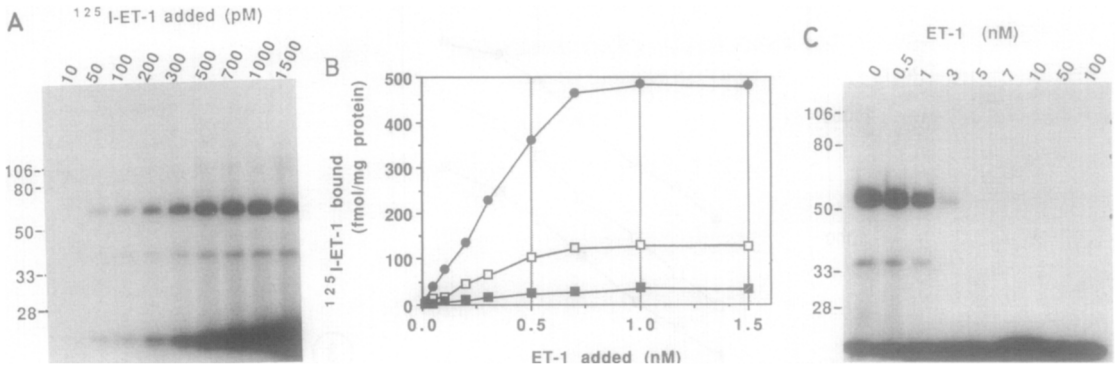

Fig. 1. Detection of the ET-1-binding protein complex with low temperature SDS-PAGE. (A) Dose dependent saturation of ET-1 binding to its binding protein. Various amount of [^{125}I]-ET-1 indicated above the figure was mixed with CHAPS extract (10 μg protein) from the canine cerebellum microsomal fraction. Incubation and electrophoresis were performed as described in Materials and Methods. (B) Detection of ET-1-binding protein complex with SDS-PAGE and calcium phosphate gel assays. Each protein band in (A) (□, 55 kD band; ■, 35 kD band) was excised from the gel and its radioactivity was counted. The calcium phosphate gel binding assay (●) was performed as described in Materials and Methods. (C) Competitive inhibition of [^{125}I]-ET-1 binding by unlabeled ET-1. 0.1 nM of [^{125}I]-ET-1 (5,000 cpm) and various concentration of unlabeled ET-1 indicated above the figure were mixed with CHAPS extract (10 μg protein) from the canine cerebellum microsomal fraction. Incubation and electrophoresis were performed as described in Materials and Methods.

1 from the complex at an early stage of electrophoresis. The release of [^{125}I]-ET-1 from the complex in the separating gel is not significant because the bands showed no smear. The intensity of the 55kD and 35 kD bands decreased by the addition of unlabeled ET-1 (Fig. 1C), indicating that the complexes were formed specifically with ET-1.

The radioactivity of each band increased as the amount of CHAPS extract was raised. The radioactivity of each band, therefore, reflects the content of endothelin binding proteins (Fig. 2). These results clearly indicate that the present method can be applied to the quantitation of a specific endothelin binding protein. Separation at low temperature is essential, since the intensity of the bands significantly reduced when electrophoresis was performed without cooling the gel (Fig. 3).

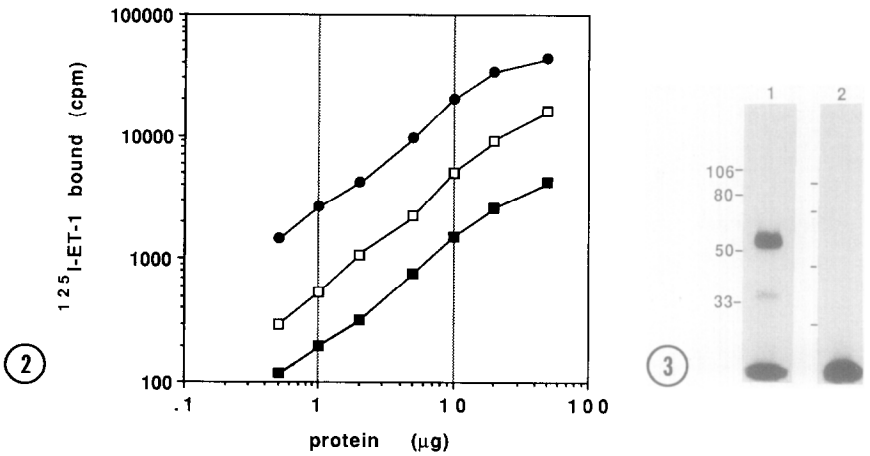

Fig. 2. Protein dose dependent complex formation between ET and its binding protein. 1 nM of [^{125}I]-ET-1 (50,000 cpm) was mixed with various amount of CHAPS extract from the canine cerebellum microsomal fraction. Incubation and electrophoresis were performed as described in the legend to Fig. 1. The calcium phosphate gel binding assay was performed as described in Materials and Methods.
●, calcium phosphate gel assay; □, 55 kD band; ■, 35 kD band.

Fig. 3. Effect of the temperature on the stability of ET-1-binding protein complex during electrophoresis. 0.2 nM of [^{125}I]-ET-1 (10,000 cpm) was mixed with CHAPS extract of canine cerebellum. Incubation and electrophoresis were performed as described in Materials and Methods. Electrophoresis was performed with cooling (lane 1) or without (lane 2).

Differences of the stability of ET-1-binding protein complexes on SDS-PAGE among animals species: In case of canine organs, clear discrete bands were detected by the method described above. We also examined human placenta and various organs of guinea pig by the same method and were successful in detecting the bands of ET-1-binding protein complex. In these samples, the presence or absence of β-mercaptoethanol did not affect the results (Fig. 4A). But, in the case of mouse organs, the signal was very weak in the presence of β-mercaptoethanol. Electrophoresis without β-mercaptoethanol, therefore, is suitable for the detection of the ET-1-binding protein complex (Fig. 4B). In addition, when the concentration of CHAPS was lowered to 0.1-0.2 % in the reaction mixture of complex formation, the amount of ET-1-binding protein complex increased in human, guinea pig and mouse samples (data not shown). These results suggest that the degree of stability of the ET-1-binding protein complex was different among the species tested.

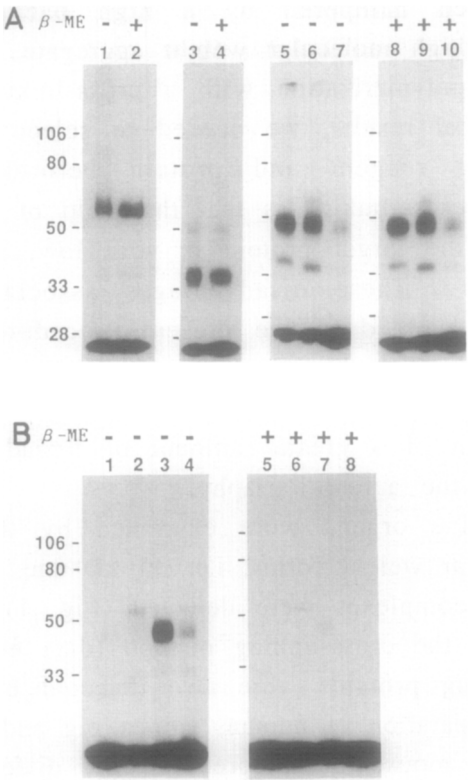

Fig. 4. Effect of β-mercaptoethanol on the autoradiographic detection of the ET-binding protein complex. (A) 0.2 nM of [^{125}I]-ET-1 (10,000 cpm) was mixed with CHAPS extract of canine cerebellum, human placenta and guinea pig organs. Incubation and electrophoresis were performed as described in Materials and Methods. Lanes 1, 3, 5, 6 and 7 were electrophoresed with the sample buffer lacking β-mercaptoethanol. Lanes: 1, 2, canine cerebellum; 3, 4, human placenta; 5, 8, guinea pig lung; 6, 9, guinea pig kidney; 7, 10, guinea pig stomach. (B) 0.2 nM of [^{125}I]-ET-1 (10,000 cpm) was mixed with CHAPS extract of mouse organs. Incubation and electrophoresis were performed as described in Materials and Methods. Lanes 1, 2, 3 and 4 were electrophoresed using sample buffer lacking β-mercaptoethanol. Lanes: 1, 5, liver; 2, 6, kidney; 3, 7, brain; 4, 8, lung.

Discussion

In this paper, we showed that the binding of [^{125}I]-ET-1 to its binding proteins could be detected simply by a cooled SDS-PAGE without cross-linking. The ET specific binding protein was detected as distinct bands after autoradiography, while non-specific binding was found to be very low. With this procedure, both qualitative and quantitative analysis are possible without cross-linking. In the case of the cross-linking experiment, the

results are often hampered by a high background and the formation of a high molecular weight aggregate, which is caused by undesirable polymerization with a cross-linking reagent. To obtain unequivocal results, we needed to select optimal amounts of cross-linking reagent and protein beforehand. Another disadvantage of cross-linking is that the yield of the incorporation of ligand with the target protein is very low, usually less than several percent. These disadvantages associated with cross-linking can be avoided in the present procedures. The yield is tenfold more than that obtained with cross-linking, and as high as 40% of the amount determined by calcium phosphate gel assay. The incorporation of a greater amount of ligand (ET), shortened the duration of the autoradiography.

When canine organs were examined by this method, two different molecular weight forms, i.e. 35 kD and 55 kD, of ET-1-binding protein complexes were detected (Fig. 1). Similar result was obtained by the cross-linking method (data not shown).

ET-1-binding proteins were also detected by this procedure in the organ preparation of human, guinea pig and mouse (Fig. 4). In the case of mouse organ preparations, different from ohter species tested, performing electrophoresis in the absence of ß-mercaptoethanol was suitable for the detection of the ET-1-binding protein complex.

Recently, cDNAs coding for the endothelin receptor of bovine lung and rat lung were cloned (20, 21). The sequence analysis of the cloned cDNA predicts that the endothelin receptor of bovine lung has a molecular weight of 48.5 kD with 427 amino acid residues (20). Under the same conditions described in this paper, we have recently analyzed the ET binding protein, extracted from bovine lung, and found that there are two classes of complex migrating at 35 kD and 55 kD (data not shown). We await further studies confirming that the 55 kD complex, detected by our low temperature SDS-PAGE, represents the receptor, the cDNA of which was recently cloned by H. Arai et. al (20). We believe that the method described here will be useful for elucidation of endothelin receptors.

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