Vol. 153, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1988 Pages 1-6

MULTIPLE FORMS OF ATRIAL NATRIURETIC FACTOR RECEPTOR IN HUMAN PLACENTA

Pampa Roy, Ulhas Naik and Indira Sen

Department of Medicine, Cardiovascular Center
Cornell University Medical College, New York, NY 10021

Received April 15, 1988

Multiple forms of atrial natriuretic factor receptor have been identified in human placental membranes. Atrial natriuretic factor binds specifically to placental membranes and the binding activity could be solubilized using non ionic detergent, Triton X-100. Binding to the detergent solubilized preparation was inhibited 80% by the addition of 0.5 M sodium chloride. Affinity cross-linking analysis indicated that this binding was associated with a single protein band of molecular weight 170-kDa. On the other hand, if sodium chloride was added together with a chelator, o-phenanthroline, ANF binding to this preparation was stimulated 300%. Binding under these conditions was not to the 170-kDa protein but was associated with a broad band in the region of 100/110-kDa and a minor band at 200-kDa. These observations clearly indicated that in human placental membranes, atrial natriuretic factor binds to distinctly different molecular species depending on the presence or absence of certain ions and chelators. The two types of binding could be conveniently assayed in the presence of each other by elimination or inclusion of sodium chloride and o-phenanthroline in the assay system.

The polypeptide hormone atrial natriuretic factor (ANF) when administered to humans or animals, exerts potent biological effects (1-4). Its actions are thought to be mediated by one or more receptors on the surface of target cells (5-13). Recently, two different forms of ANF receptor have been purified from bovine adrenal cortex which differ in pharmacological properties as well as their primary structures (5). Although multiple forms of ANF receptors have been well characterized pharmacologically and physicochemically in various animal tissues and cell lines,

ABBREVIATIONS: ANF, atrial natriuretic factor; SDS, sodium dodecyl sulfate.

0006-291X/88 $1.50

very little is known about the characteristics of ANF binding to human tissues. We have reported previously, that membranes prepared from human placenta binds ^{125}I-ANF specifically (14) and the binding activity can be solubilized by treatment with Triton X-100. Here we describe some properties of the solubilized binding activity which suggests that multiple forms of ANF receptor exist in human placenta.

EXPERIMENTAL PROCEDURES

Preparation of Membranes from Human Placenta and Solubilization. The homogenization of fresh placentas from full-term deliveries and isolation of particles sedimenting between 12,000 and 40,000 x g has been previously described (14). This pellet was washed three times with 50 mM potassium phosphate buffer pH 7.5 containing 0.5 mM phenylmethylsulfonyl fluoride and suspended in the same buffer at a protein concentration of 5-6 mg/ml. The solubilization of ANF binding activity was carried out as described before by extraction with 1% (v/v) Triton X-100. After centrifugation at 105,000 x g for 60 min, the supernatant fluid was collected and used as the Triton X-100 extract. Both the membrane preparation and the solubilized extract were kept frozen at -70°C for at least three months without loss of ANF binding activity.

ANF-binding activity. The complete assay system, in a final volume of 100 µl, contained 10-20 µg of receptor preparation, 20 mM potassium phosphate buffer, pH 7.5, 0.1% bovine serum albumin, 1.0 mM phenylmethylsulfonyl fluoride and ^{125}I-ANF (99-126), 50,000 cpm (Amersham). Sodium chloride (0.1 - 0.5 mM), EDTA (5 mM), $MgCl_2$ and/or $MnCl_2$ (1-10 mM) and o-phenanthroline (1 mM) were added as required. After an incubation of 90 min on ice the protein bound hormone was separated from unbound hormone by adsorption on 0.25% charcoal coated with 1% Dextran as described before (15). The trapped radioactivity in the charcoal-dextran supernatant was measured in a gamma counter. In duplicate assays, unlabeled ANF (1 µM) was added to determined non specific binding.

Affinity cross linking of ^{125}I-ANF to the ANF receptor. The solubilized extracts were incubated with ^{125}I-ANF as described above (except that bovine serum albumin was eliminated from the incubation) with or without 10 µM ANF. After 90 min at 0°C, the incubation was transferred to 4°C and the bifunctional cross-linking reagent disuccinimidyl suberate (Pierce) in dimethyl-sulfoxide was added at a final concentration of 0.2 mM. Fifteen minutes later, the reaction was quenched by the addition of 2 M ammonium acetate (final concentration 50 mM) and heated to 100°C for 3 min after the addition of SDS-stopping buffer giving a final concentration of 1% SDS (w/v) 20% glycerol (v/v), 12.5 mM Tris/HCl, pH 6.8 and 5% 2-mercaptoethanol (v/v). The samples were then analyzed by electrophoresis on 5-15% gradient SDS-polyacrylamide gels and radioautographed as described before (14).

Protein was determined by the Bio-Rad assay (16) using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Some characteristics of binding of ANF to the Triton X-100 solubilized placental membranes are shown in Table 1. Ninety-five percent of the binding was displaced by 1 μM ANF but was unaffected by unrelated hormone like insulin. NaCl, $MgCl_2$ and $MnCl_2$ when added individually to the assay mixture were found to inhibit binding 69 - 84%, whereas, EDTA and o-phenanthroline increased the binding 1.6 and 2.7 fold, respectively.

In order to further characterize the binding activity, the effect of NaCl and o-phenanthroline was studied in more detail. It was observed that NaCl plays a dual role in the binding of ANF to its receptor in presence and absence of o-phenanthroline (Fig. 1). In absence of o-phenanthroline, the binding decreased as the NaCl concentration was increased, the inhibitory effect reaching a maximum between 0.3 - 0.5 M. On the other hand, in the presence of o-phenanthroline, ANF binding increased by increasing the concentration of NaCl reaching a maximum at 0.5 M. This opposite effect of NaCl in which the ion either decreased binding by 85% or increased it by 300%, strongly suggested that ANF might be binding to different types of receptor protein under these two conditions.

To obtain structural information about the receptors, ^{125}I-ANF was allowed to bind to the preparation under the following three conditions; (a) the complete system, or with additives, (b)

Table 1. Binding characteristics of the solubilized fraction

System	Binding cpm
Complete	2000
+ ANF (1 μM)	110
+ Insulin (1 μM)	1980
+ NaCl (300 mM)	320
+ $MgCl_2$ (10 mM)	620
+ $MnCl_2$ (10 mM)	400
+ $MgCl_2$ (10mM) + $MnCl_2$ (5 mM)	500
+ EDTA (5 mM)	3200
+ o-phenanthroline (1 mM)	5400

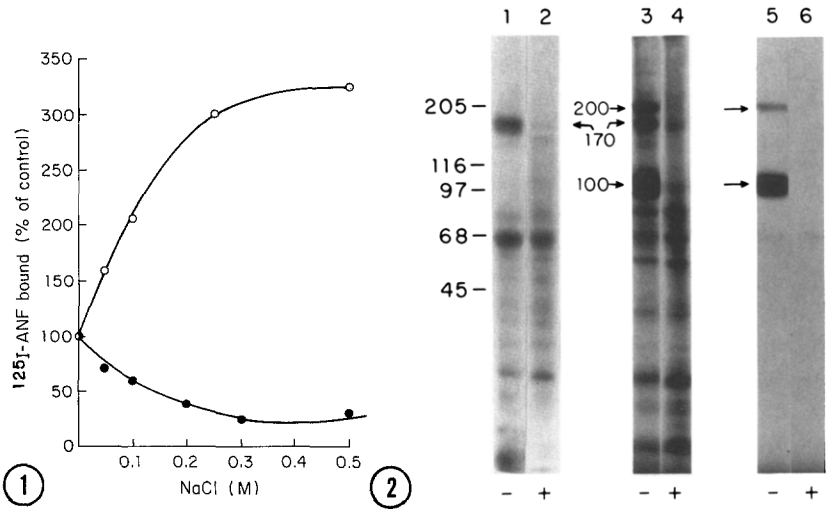

Fig. 1. Effect of sodium chloride on ANF binding. Triton X-100 solubilized preparations (10-12 µg) were incubated with ANF as described under experimental conditions in presence (o) or absence (●) of 1 mM o-phenanthroline. NaCl concentration was varied from 0 to 0.5 M as indicated. The specific control binding (i.e., at zero NaCl concentration) was 2000 cpm in absence and 5500 cpm in presence of o-phenanthroline.

Fig. 2. Specific cross-linking of ^{125}I-ANF to its binding proteins. Solubilized preparation (10 µg) was incubated with ^{125}I-ANF as described in the experimental procedures, under three different conditions: Lanes 1 and 2, with no extra additives, Lanes 3 and 4, with 0.1 M NaCl and 1 mM o-phenanthroline and lanes 5 and 6, with 0.5 M NaCl and 1 mM o-phenanthroline. The lanes marked + (2, 4 and 6) and - (1, 3 and 5) are with and without additional 1 µM cold ANF. After incubation on ice for 90 min the bound ^{125}I-ANF was covalently cross-linked to its receptors by disuccinimidyl suberate and analyzed by electrophoresis followed by autoradiography. The position of the protein markers used (purchased from Bio-Rad) are indicated on the left. They are: myosin, 205-kDa; β-galactosidase, 116-kDa; phosphorylase b, 97-kDa; bovine serum albumin, 68-kDa, and ovalbumin, 45-kDa.

0.1 M NaCl and 1 mM o-phenanthroline, or (c) 0.5 M NaCl and 1 mM o-phenanthroline. The receptor ligand complex was then treated with disuccinimidyl suberate to covalently cross-link the ligand to its receptor and analyzed by SDS-polyacrylamide gel electrophoresis followed by radioautography under reducing conditions. It was observed, that only one prominent radioactive band corresponding to the molecular mass of 170-kDa could be seen in the complete system, the intensity of which was greatly reduced by the inclusion of unlabeled ANF in the original incubation (Fig. 2, lanes 1 and 2).

In the presence of o-phenanthroline and 0.5 M NaCl, on the other hand the 170-kDa band was not detectable, but, two other prominent radioactive bands appear which are also displaced completely by the addition of excess unlabeled ANF. These are, a broad band at 100-kDa and a relatively minor band at 200 kDa (Fig. 2, lanes 5 & 6). When the sodium chloride concentration was 0.1 M, all three bands are visible (Fig. 2, lane 3). Several other radioactive bands are also visible in lanes 3 and 4 but none of these are specifically displaced by unlabeled ANF. Densitometric analysis of the radioautograms indicated that the radioactivity incorporated in the 100 kDa band is 20 times more than in the 200-kDa band. The broad band at 100-kDa is actually a doublet with two bands at 100-kDa and 110-kDa which does not separate very well under these conditions. The above results clearly indicated that in human placental membranes, ANF binds to more than one type of receptor. In the presence of o-phenanthroline and high concentration of sodium chloride, ANF binds to a 100/110-kDa receptor protein with a minor amount binding to a 200-kDa band. In its absence, the binding is solely to the 170-kDa receptor.

The binding in presence of o-phenanthroline and 0.5 M NaCl was much more abundant than in its absence. Analyses of two successive 200 g placentas showed 4361 and 4572 p moles of specific binding activity in presence of the chelator and 0.5 M NaCl, whereas, only 459 and 300 p moles of specific binding was detected in their absence indicating that the 170-kDa receptor is a relatively minor species and the 100/110-kDa form is the most abundant form. In conclusion, this is the first demonstration of the presence of multiple forms of ANF receptors in human tissue.

ACKNOWLEDGEMENT This work was supported by National Institutes of Health Grants 3P50-HL18323, ROI HD 22526 and an American Heart Association Grant-in-Aid.

REFERENCES

1. Maack, T., Camargo, M.J.F., Kleinert, H.D., Laragh, J.H. & Atlas, S.A. (1985) Kidney Int., 27, 607-615.
2. Genest, J. & Cantin, M. (1987) Circulation, 75 (Suppl. 1), I - 118.
3. Needleman, P. et al (1987) Hypertension Dallas, 7, 469-482.
4. Atlas, S.A., Laragh, J.H. (1987) Atrial Hormones and Other Natriuretic Factors, 53-75, American Physiological Society, Bethesda.
5. Takayanagi, R., Inagami, T., Snajdar, R.M., Imada, T., Tamura, M. & Misono, K.S. (1987) J. Biol. Chem., 262, 12104-12113.
6. Yip, C.C., Laing, L.P., and Flynn, T.G. (1985) J.Biol Chem., 260, 8229-8232.
7. Shimonaka, M., Saheki, T., Hagiwara, H., Ishido, M., Nogi, A. Fujita, T. Wakita, K., Inada, Y., Kundo, J. & Hirose, S. (1987) J. Biol. Chem., 262, 5510-5514.
8. Schenk, D.B., Phelps, M.N., Porter, J.G., Fuller, F., Cordell, B. & Lewicki, J.A. (1987) Proc. Natl. Acad. Sci. USA, 84, 1521-1525.
9. Vandlen, R.L. Arcuri, K.E., and Napier, M.A. (1985) J. Biol. Chem., 260, 10889-10892.
10. Meloche, S., Ong, H., Cantin, M. and De Lean, A. (1986) J. Biol. Chem., 261, 1525-1529.
11. Kuno, T., Andresen, J.W., Kamisaki, Y., Waldman, S.A., Chang, L.Y., Sakeki, S., Leitman, D.C., Nakana, M. & Murad, F. (1986) J. Biol. Chem., 261, 5817-5823.
12. Leitman, D.C., Andresen, J.W., Kuno, T., Kamisaki, Y., Chang, J.K. & Murad, F. (1986) J. Biol. Chem., 261, 11650-11655.
13. Paul, A.K., Marala, R.B., Jiswal, R.K. & Sharma, R.K., (1987) Science, 235, 1224-1226.
14. Sen, I.,(1986) Biochem. Biophys. Res. Commun., 135, 480-486.
15. Sen, I., Jim, K.F. and Soffer, R.L. (1983) Eur. J. Biochem., 136, 41-49.
16. Bradford, M. (1976) Anal. Biochem., 72, 248-254.