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# A SOLUBLE ANGIOTENSIN II-BINDING PROTEIN FROM RABBIT LIVER

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An angiotensin II-binding activity has been detected in the 100,000 x g supernatant fraction of rabbit liver. The total amount of binding activity in this fraction was substantially greater than that which could be solubilized from hepatic particles by treatment with digitonin. The crude soluble binding activity resembled the binding protein which had been purified from the particles in several respects. First, binding required the presence of p-chloromercuriphenylsulfonic acid and bound angiotensin II was released by dithiothreitol. Second, the molecular weight of the responsible protein cross-linked to radioiodinated angiotensin II was about 75,000 in the reduced, denatured state. Finally, guinea pig antiserum raised against the binding protein that had been purified from particles reacted identically with the soluble and solubilized activities. 

Angiotensin II is the effector molecule of the renin-angiotensin system (1). Its actions are thought to be mediated by one or more receptors on the surface of target cells (2,3). We recently purified an angiotensin II-binding protein after solubilization from rabbit hepatic particles (4). This protein appears to be a physiologically important angiotensin receptor since antibodies against it inhibited the vasopressor response of rabbit renal arteries to angiotensin II (5). We have now noticed that most of the angiotensin II-binding activity of a rabbit liver homogenate is present in the 100,000 x g supernatant fraction. Here we describe some properties of this crude, soluble binding activity which suggests that it is due to a protein resembling closely that which we purified from particles. The unexpected presence of a cytosolic binding protein suggests that some actions of angiotensin II may be mediated intracellularly.

## MATERIALS AND METHODS

Materials - [$^{125}$I] Angiotensin II (1400 μCi/nmol) and Ile$^{5}$-angiotensin II were from New England Nuclear and Vega

Abbreviation: PCMS, p-chloromercuriphenylsulfonic acid.

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Biochemicals. HCl-activated charcoal, digitonin, PCMS, arginine$^8$-vasopressin and porcine insulin were Sigma products. Brij 99 and disuccinimidyl suberate were from ICI and Pierce. Reagents for electrophoresis were from Bio-Rad. Pharmacia products included Sephadex G-50, Dextran T70 and protein A-Sepharose CL-4B. The latter was made into a slurry (100 mg/ml) with 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 100 mM NaCl.

Homogenization and tissue fractionation - Freshly excised New Zealand White rabbit livers were homogenized in a Waring blender with three volumes of chilled 50 mM Tris-HCl, pH 7.5, containing 1 mM EDTA for three 45 second periods punctuated by five minute intervals in a water-ice bath. The homogenate was passed through two layers of cheese cloth and centrifuged, first for 15 min at 1000 x g, then for 90 min at 100,000 x g. The latter pellet was dispersed in an equal volume of buffer and recentrifuged at 100,000 x g. The two supernates were combined as the soluble (100,000 x g supernatant) fraction. The pellet was suspended and stirred overnight in three volumes of buffer containing 1% digitonin and the crude solubilized fraction was obtained by centrifugation at 100,000 x g.

Standard binding assay - Reaction mixtures (150 µl) contained 30 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 0.2 mM PCMS, 100 µg bovine serum albumin, 0.2 nM [$^{125}$I) angiotensin II (80,000 cpm), 19.8 nM unlabeled angiotensin II and binding protein. Brij 99 (0.25%) was included for reaction mixtures containing solubilized binding protein. Reactions were carried out for 60 min at 20° and bound radioactivity counted in the supernatant fraction after the samples had been treated with 0.05/0.5% w/v dextran/charcoal and centrifuged (4). Results were corrected for nonspecific binding in the presence of unlabeled 10 µM angiotensin II. This correction was insignificant (< 10%) except for binding to the crude solubilized fraction where it was 40-60%.

Cross-linking - Reaction mixtures (300 µl) contained 30 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 0.2 mM PCMS, [$^{125}$I] angiotensin II (1x10$^6$cpm), 300 µg soluble protein and either 0 or 10 µM unlabeled angiotensin II. After 60 min at 20°, they were filtered through columns (27 x 0.7cm) of Sephadex G-50 in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA. The two void volumes contained 4.4 x $10^5$ and 5.4 x $10^3$ cpm. Proportional aliquots of each (7.9 x $10^4$ and 1 x $10^3$ cpm) were treated with 2% dimethyl sulfoxide and incubated 15 min at 4° in the presence or absence of 0.4 mM disuccinimidyl suberate. They were then immediately reduced, denatured and subjected to electrophoresis (6) through 5-15% polyacrylamide gradients. The autoradiograms were developed after exposure to Kodak X-Omat AR film for 90 hr at -70°.

Immunological procedures - A 700 gram Hartley guinea pig was immunized intradermally six times at 2 - 3 week intervals with 6 µg of binding protein which had been purified through the hydroxylapatite step after solubilization from rabbit hepatic particles (4). The preparation bound 8.4 nmoles of angiotensin II per mg and its purity was about 60%. The animal was bled three days after the last injection and the antiserum was heated at 56° for 30 min. In assays for removal of binding activity, the antiserum was shaken for 2 hr at 20° in a reaction mixture (30 µl) containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 1 mg of protein A-Sepharose and 0.5% Brij 99. The slurries were washed, centrifuged and the pellets incubated for 16 hrs at 4° with 70 µl of crude soluble and partially purified (through Sephacryl S-200 step of our procedure [4]) solubilized binding activities in the same buffer. After centrifugation, the supernates were assayed for residual binding activity under the standard assay conditions

except that unlabeled angiotensin II was omitted and Brij 99 was present at 0.25%. Amounts of binding protein were selected so that the 100% value (reaction mixture lacking serum) was 8,000 cpm. Direct inhibition of binding activity was also assayed under these conditions.

Determination of protein content - The method of Lowry et al (7) was employed with bovine serum albumin as a standard.

## RESULTS

A typical time and dose-response for the binding of angiotensin II by the soluble fraction is shown in Figure 1. Binding reaches a plateau value within 60 min at which time soluble fractions from the livers of different animals bound 2-3 pmoles of angiotensin II per mg protein. The soluble fraction accounted for the vast majority of recovered binding activity. For example, analyses of three successive 70 - 75 g livers revealed 16592, 16705 and 14033 pmoles of specific binding activity in the soluble fractions, whereas only 762, 490 and 465 pmoles could be extracted from the washed 1,000-100,000 x g pellets with digitonin i.e. more than 95% of the recovered specific binding activity was present in the soluble fraction. The soluble nature of the responsible protein in this 100,000 x g supernatant fraction was confirmed by glycerol gradient

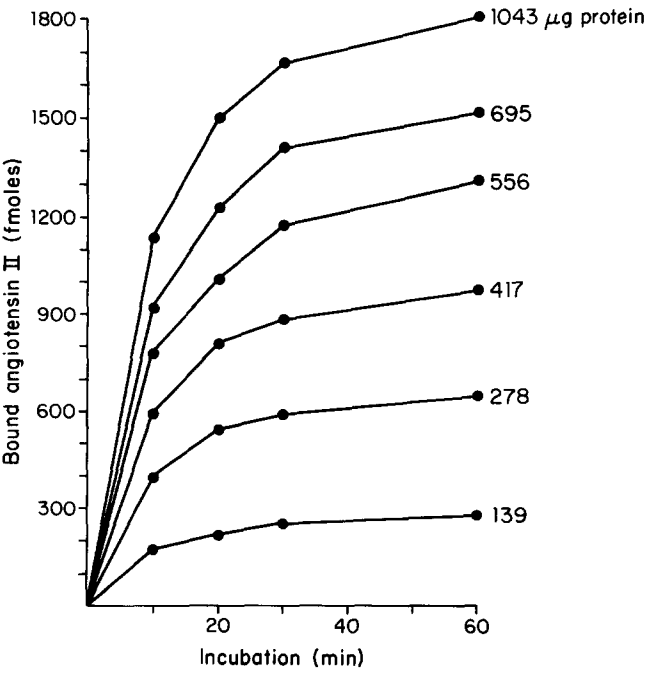


Fig. 1. Binding of angiotensin II by the soluble fraction. Reactions were carried out using the standard binding assay described under METHODS.

Table 1. Binding of radioiodinated angiotensin II by the soluble fraction

| System | Binding (cpm) |
|---|---|
| Complete[a] | 19903 |
| -Soluble fraction | 300 |
| + 10 μM Angiotensin II | 800 |
| + 10 μM Vasopressin | 19297 |
| + 10 μM Porcine insulin | 19709 |
| - PCMS | 679 |
| + 1 mM Dithiothreitol[b] | 873 |

[a] The standard binding conditions were employed with 340 μg of soluble protein.

[b] Added after the standard 60 min incubation and reaction mixture processed after additional 10 min.

centrifugation (not shown) during which the binding activity sedimented as a single peak just ahead of albumin.

Some characteristics of the binding reaction are shown in Table 1. The binding of [$^{125}$I]angiotensin II was abolished by 10 μM unlabeled angiotensin II, but not affected by the presence of unrelated peptides. It was almost completely dependent on PCMS and bound radioactivity was released by dithiothreitol. These unusual properties are also exhibited by the protein previously purified from particles (4,8). Evidence obtained with that protein indicates that it contains a critical sulfhydryl group which must be covalently linked with the organomercurial to generate and maintain an active binding configuration (4).

Cross-linking of [$^{125}$I]angiotensin II to the crude cytosol is shown in Figure 2. There was only one radioactive polypeptide whose appearance was blocked by unlabeled angiotensin II or omission of disuccinimidyl suberate. The apparent molecular weight of this soluble protein in the reduced, denatured state, 75,000 daltons, corresponds identically to the value found for the protein purified from particles (4). The gel also shows a radioactive band with the mobility of albumin which appears to be a trace contaminant of the [$^{125}$I]angiotensin II preparation.

Figure 3 presents data obtained with guinea pig antiserum that had been developed against an almost pure preparation of solubilized binding protein from particles. The antibody dose-response curves for removal of binding activity from solution in the presence of protein A-Sepharose and for direct inhibition of binding activity were virtually identical for the crude soluble

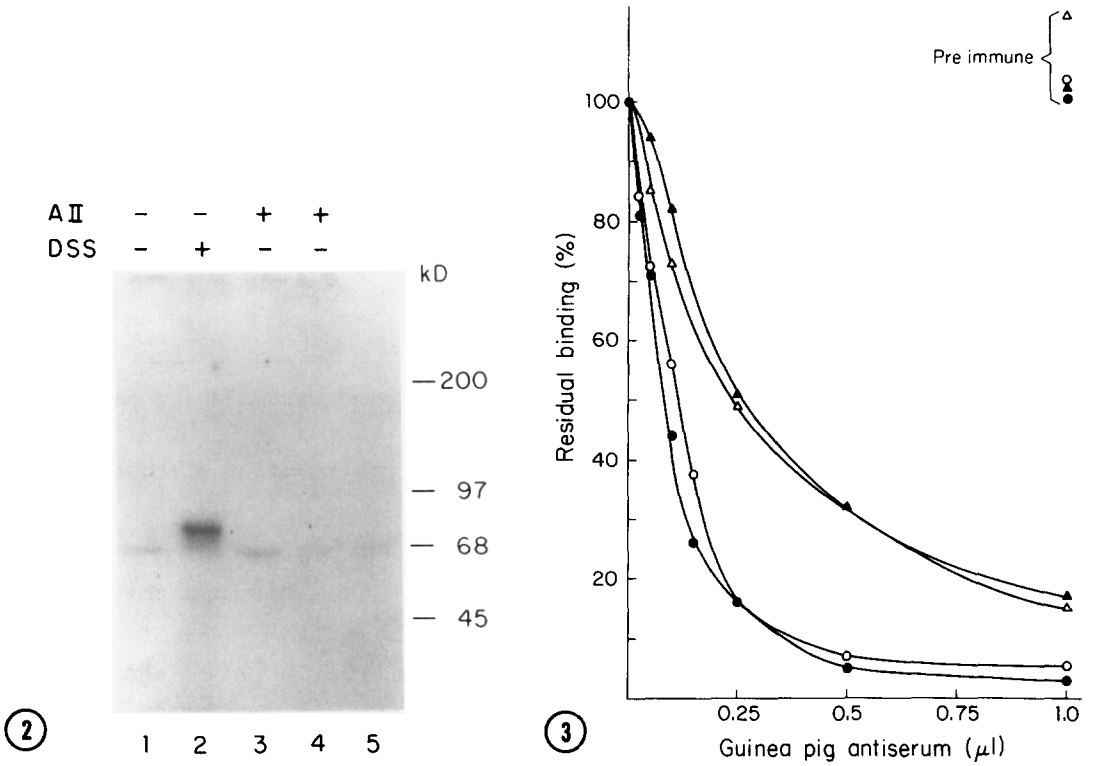


Fig. 2. Gel electrophoresis of cross-linked angiotensin II. [$^{125}$I]Angiotensin II was reacted with the soluble fraction and processed as described under METHODS in the presence and absence of disuccinimidyl suberate (DSS) and unlabeled angiotensin II (AII). Lane 5 contained only the [$^{125}$I]angiotensin II (79,000 cpm) preparation.

Fig. 3. Immunological analysis of the angiotensin II-binding activities. Guinea pig antiserum against the binding protein purified from particles was used for removal of binding activity (circles) and for its direct inhibition (triangles) as described under METHODS. Open and closed symbols denote the solubilized and soluble activities.

fraction and for a partially purified preparation of the solubilized receptor. These results indicate a very high degree of immunological homology between the responsible soluble and solubilized proteins.

## DISCUSSION

This communication describes evidence for the presence of a cytosolic angiotensin binding protein which resembles closely the angiotensin II receptor that we solubilized and purified from particles (4). The similarity of the soluble and solubilized binding proteins is indicated by their requirement for PCMS, by their similar molecular weights and by their immunological homology. Characterization of the ligand specificity and

quantitative binding parameters of the soluble protein will require its purification. However, it does not appear to be a particularly scarce molecule as suggested by the following consideration. The crude cytosol binds 2-3 pmoles of angiotensin II per mg under the standard assay conditions, which, in the case of the purified receptor (4) yields a figure 70% that of the $B_{max}$ value. Based on a correction for this and the assumption that the responsible 75 kilodalton protein binds one mole of angiotensin II, the purification factor required to achieve homogeneity can be roughly calculated as 3,000 - 4,500 i.e. the soluble binding protein constitutes about 0.02 - 0.03% of total cytosolic protein. A similar calculation suggests that a single 70 gram rabbit liver may contain about 1-2 mg of this protein. It seems to us unlikely that such a large amount of this binding activity could derive from "internalization" of a surface receptor, particularly since the cytosol contains so much more of it (Ca. 20-fold) than the particles. This protein is apparently not restricted to the liver since we have detected (unpublished observations) an immunologically similar binding activity in the cytosol of various other rabbit organs including brain, aorta, kidney, uterus and adrenal grands.

What then is this protein doing in the cytosol? It seems possible to us that angiotensin II, in addition to its immediate effects at the cell surface, may also act by a slower intracellular mechanism. Perhaps this protein transports angiotensin II to an important internal site or perhaps it mediates the action of internally produced angiotensin II. In these contexts it is interesting, first, that an early autoradiographic study suggested the accumulation of radioactive angiotensin II within the nuclei of cardiac and vascular myocytes (9), and second, that at least certain cell types are capable of synthesizing their own angiotensin II (10,11).

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