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Rat Liver AT_1 Receptor Binding Analysis for Drug Screening

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Abstract—The only compounds with antagonistic activity via AT_1 receptor, one of two subtypes of angiotensin II (AII) receptor, have been demonstrated to block the vasoconstriction effects of AII and thereby provide therapeutic potential. This initiated the search for compounds with high specific affinity to AT_1 receptor and their effective screening methods. The radioligand binding assay for the AII receptor is regarded as the primary method for the evaluation of AT_1 receptor antagonists for their activity. In this paper, we characterized the liver AT_1 receptor and describe the efficient method of the radioligand binding assay using rat liver as a source of AT_1 receptor. Equilibrium binding studies with rat adrenal cortex, adrenal medulla, liver and bovine adrenal showed that the specific bindings of [^{3}H] AII were saturable in all tissues and the Scatchard plots of those data were linear, suggesting a single population of binding sites. Hill slopes were very near to the unity in all tissues. Kinetic studies of [^{3}H] AII binding in rat liver homogenates yielded two association rate constants, $4.10\times10^7\ M^{-1}min^{-1}$ and $4.02\times10^9\ M^{-1}min^{-1}$, with a single dissociation rate constant, $7.07\times10^{-3}\ min^{-1}$, possibly due to the partial dissociation phenomenon. The rank order of inhibition potencies of [^{3}H] AII binding in rat liver was AII>Sarile>Losartan>PD 123177. Rat liver homogenates revealed to have very high density of homogeneous population of the AT_1 receptor subtype, as the specifically bound [^{3}H] AII was not inhibited by PD 123177, the nonpeptide antagonist of AT_2. The results of this study demonstrated that the liver homogenates from rats could be the best receptor preparation for the AT_1 receptor binding assay and provide an efficient system for the screening of newly synthesized candidate compounds of AT_1 receptor antagonist.

Keywords □ rat liver, angiotensin II, AT_1, AT_2, drug screening, receptor binding

The peptide hormone Angiotensin II (AII) exerts its numerous physiological actions through binding to membrane receptors. At least two subtypes of receptor, AT_1 and AT_2, for AII exist, which can be distinguished on the basis of their different affinity for synthetic ligands. The non-peptide antagonist DuP 753 (Losartan) is specific for the AT_1 receptor subtype, while the synthetic peptide CGP 42112 and non-peptide PD 123177 are specific for the AT_2 receptor subtype and these compounds do not have the cross-reactivity between AT_1 and AT_2. The binding of radiolabeled AII to AT_1 receptors is reduced with DTT (Speth *et al.*, 1991; Whitebread *et al.*, 1989), whereas binding to AT_2 receptors is either enhanced or unaffected (Speth *et al.*, 1991; Whitebread *et al.*, 1989). Recently, it has been proposed that both AT_1 and AT_2 receptors can be further subdivided into AT_{1a}, AT_{1b}, AT_{2a} and AT_{2b} based on pharmacological profiles and molecular biological approaches (Iwai and Inagami, 1992; Tsutsumi and Saavedra, 1992). Also, [^{3}H] DuP 753 binding to rat liver homogenates reveals the presence of a large population of nonangiotensin binding sites (Widdowson *et al.*, 1993).

The expression of these two subtypes is tissue-specific and species-specific : some tissues express only one subtype while others express both (Timmermans *et al.*, 1993). Most known physiological actions of AII, such as regulation of blood pressure, extracellular fluid volume, catecholamine and aldosterone secretion, etc., can be ascribed to its binding to the AT_1 receptor (Wong *et al.*, 1990; Dudley *et al.*, 1990). However, little is known about the AT_2 receptor subtype, but apparently it also comprises different molecular properties in bovine cerebellum (Bottari *et al.*, 1991), human myo-

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metrium (Bottari *et al.*, 1991; Whitebread *et al.*, 1989; Whitebread *et al.*, 1991; Lazard *et al.*, 1994) and rat ovarian granulosa cells (Pucell *et al.*, 1991).

As DuP 753 and other imidazole-based compounds described in the DuPont de Nemours patent (EPA 253 310, January 1988) effectively lowered the blood pressure in renal artery ligated rats (a high renin model), whereas PD 123177 was ineffective in reducing blood pressure. The conclusion was that only compounds with AT_1 antagonistic activity were able to block the vasoconstriction effects of AII and thereby provide therapeutic potential. This initiated the search for other compounds with high specific affinity to AT_1 receptor and their effective screening methods. Among those methods, the radioligand binding assay for the AII receptor is regarded as the primary one for the evaluation of the binding affinity of AT_1 receptor antagonist. Various tissues were tested as a part of the AII receptor research and for the drug screening purpose, however, there have been lots of technical difficulties to get the enough amount of receptor preparation for the screening of hundreds of newly synthesized compounds.

In this paper, we describe the efficient method of the AT_1 receptor preparation from rat liver and the characterization of the liver AT_1 receptor through equilibrium binding, kinetic analysis and competition experiments with standard compounds. The reason for selecting rat liver as the AT_1 receptor source is that the highest densities of the AT_1 receptor subtype is found in vascular muscle and liver, while the adrenal gland and brain contain a mixture of both AT_1 and AT_2 types (Chiu *et al.*, 1989; Crane *et al.*, 1982; Dudley *et al.*, 1990; Gibson *et al.*, 1991; Whitebread *et al.*, 1989).

Upon elucidating the nature of the liver AT_1 receptor, the radioligand binding analysis can be more effective and stable tool as one of the primary screening system.

Materials and Methods

Materials

[^{3}H]AII(5-L-isoleucine, 65 Ci/mmole) was purchased from DuPont NEN(Boston, MA). AII(human), Saralasin and [Sar^1, Ile^8] AII were purchased from Sigma (St. Louis, MO). Lumagel scintillation cocktail was obtained from Lumac*LSC B.V. (Olen, Belgium). The nonpeptide angiotensin antagonists, DuP 753 [(2-n-butyl-4-chloro-5-hydroxymethyl-1- [2'(1H-tetrazol-5-yl)biphenyl-4-yl)-methyl]imidazole] and CV-11974 [2-Ethoxy-1-[[2'-(1 H-tetrazol-5-yl)biphenyl-4-yl]methyl]-1H-benzimidazole-7-carboxylic acid], were synthesized in the laboratory of Dr. Sungeun Yoo, Korea Research Institute of Chemical Technology(KRICT). The PD 123177 [1-(4-amino-3-methylphenyl)-methyl-5-diphenylacetyl-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid-2HCl] was synthesized in Hanhyo Institute of Technology. All other chemicals were of the highest purity commercially available.

Preparation of the Particulate Membrane Fraction from Rat Liver

Male Sprague-Dawley rats (300~400 g) were supplied by Animal Research Lab., KRICT and kept on the standard laboratory chow. AII receptors from liver microsomes were prepared by minor modifications of the couple of methods (Glossmann *et al.*, 1974; Gunther *et al.*, 1980).

Livers were obtained after cervical dislocation and kept in 10~50 volumes of ice-cold sucrose buffer containing 0.2 M sucrose, 1 mM EDTA and 10 mM Trizma base (pH 7.2). After mincing and rinsing with same buffer, the tissues were disrupted with Brinkmann Homogenizer (Brinkmann Instruments, Inc.). The homogenate was spun at 3,000×g for 10 min and supernatant was decanted through KimWipes. Combined supernatants were spun at 12,000×g for 13 min. The final supernatant was then centrifuged at 102,000×g for 60 min.

The pellet was washed with washing buffer containing 5 mM $MgCl_2$, 50 mM Trizma base (pH 7.2). All of the previous steps were carried out at 4℃. After resuspending the final pellet in washing buffer, the content of protein was adjusted to 1.5~2.0 mg/m*l* using Biorad DC protein assay kit.

The final suspensions in assay buffer containing 0.25 % bovine serum albumin (BSA), 5 mM $MgCl_2$ and 50 mM Trizma base (pH 7.2) were divided into 4 m*l* aliquots and then frozen at −80℃. The protein aliquots were stored at −80℃ until used.

AII binding to Rat Liver Homogenates

Binding assays were performed in triplicate tubes by incubating aliquots of freshly prepared particulate fraction (0.15~0.20 mg of protein) with varying concentrations of [^{3}H] AII with or without inhibitor in 13× 100 mm borosilicated glass tubes in a final volume of 0.5 m*l* of assay buffer. Under these experimental conditions, the binding of [^{3}H] AII was linear with time and with increasing protein concentration.

After incubation in a shaking water bath for 60 min at 25℃, the reaction was terminated by the addition of 3 m*l* of cold washing buffer and the bound/free radioactivity was separated rapidly through galss fiber

filters (GF/C Whatman, presoaked with assay buffer) with a Brandel cell harvester system (Brandel M-12R).

The filters were washed with an additional 3 ml of cold washing buffer and trapped radioactivity was measured by a Packard scintillation counter (Packard Tricarb 1500C).

All test compounds were dissolved at 1 mM in dimethylsulfoxide (DMSO) and serially diluted to various concentrations for activity screening. All data presented are specific binding, defined as that displaceable by 1 μM unlabeled AII added to the assay mixture.

Data Analysis

Equilibrium binding parameters and binding isotherms from kinetic studies were obtained using the iterative nonlinear curve fitting program LIGAND (Munson and Rodbard, 1980). The inhibitory concentration (IC_{50}) of an inhibitor that gave 50% displacement of the specific binding of labeled AII was determined by linear regression analysis of the displacement curve converted through logit transformation. The single-site and two-site models for each isotherm were calculated using the differential F value in the LIGAND program.

Results

Binding Studies of [^{3}H]AII in Various Tissue Preparations

To compare the binding properties of [^{3}H]AII in various tissues, microsomal fractions were prepared by the differential centrifugation described in methods section. The assay condition for all preparations was identical (60 min incubation at 25℃). The protein contents in particulate fractions were adjusted to different ranges in binding experiments (rat adrenal cortex : 0.02~0.03 mg protein, rat adrenal medulla : 0.15~0.2 mg protein, bovine adrenal : 0.08~0.12 mg protein). Those protein ranges for various tissues were best fitted to the study of [^{3}H] AII binding as determined by the protein dose analysis (data not shown) in the range of 0.01 to 0.5 mg of protein to confirm the linearity of [^{3}H]AII binding with the increasing protein concentration.

In equilibrium binding analysis, the saturability of specific binding was found in rat adrenal cortex, medulla and bovine adrenal. The Scatchard plots of those data showed linear distribution suggesting an interaction of the ligand with a single population of sites and it was confirmed by the data analysis through the LIGAND program. The range of the dissociation constant (K_d) was 1.35~2.97 nM and an apparent maximum binding (B_{max}) was 172~950 fmol/mg protein at various concentrations of [^{3}H] AII (0.1~10 nM) under the experimental condition of 60 minutes incubation at 25℃ (Table I). Hill slopes were very near to the unity in all tissues. The competition binding studies with standard materials were also demonstrated in Table I. The IC_{50} values of Losartan were similar to each other using receptor preparations of rat adrenal cortex and liver. The receptor preparation from bovine adrenal showed higher IC_{50} value with Losartan or Saralasin than that of other tissues and this might be due to the poor separation of cortex and medulla, as it is not easy to separate them as in the rat adrenal.

Equilibrium Studies of [^{3}H]AII Binding in Rat Liver Homogenates

Saturation experiments with [^{3}H]AII (0.1~10 nM) showed binding to a single site in rat liver homogenates with the dissociation constant (K_d) of 2.56± 0.85 nM (mean± S.E.) and an apparent maximum binding (B_{max}) of 352± 7.22 fmol/mg protein under the experimental condition of 60 minutes incubation at 25℃ (Fig. 1). The slope of Hill plot (Hill coefficient, nH) was 0.9999± 0.001 suggesting an interaction of the ligand with a single population of binding sites (Table I).

For the analysis of the mode of AII receptor antagonistic action in rat liver, equilibrium binding experime-

Table I. Equilibrium binding parameters in various tissues

Receptor Source	K_d (nM)	B_{max} (fmol/mg of protein)	Hill Slopes	IC_{50} (M)
Rat Adrenal Cortex	1.35	950	0.9849	Losartan : 6.65×10^{-8} Saralasin : 6.98×10^{-9}
Rat Adrenal Medulla	2.97	172	0.9959	N.D.[a]
Bovine Adrenal	2.84	256	0.9974	Losartan : 1.85×10^{-7} Saralasin : 2.50×10^{-8}
Rat Liver	2.56	352	0.9999	Losartan : 8.04×10^{-8} Sarile : 1.35×10^{-8} AII : 3.18×10^{-9}

The concentrations of [^{3}H]AII for competition were 3 nM in rat liver and 2 nM in other tissues. These data were calculated as described in the section of data analysis. [a]N.D., not determined.

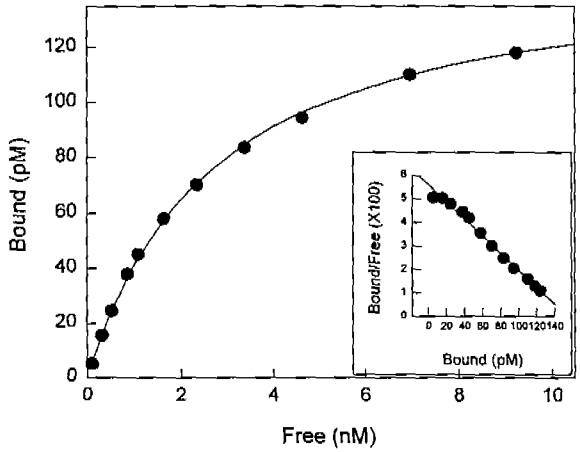

Fig. 1. Binding of [^{3}H]AII (0.1~10 nM) to rat liver homogenates. Inset shows a Scatchard plot of the same data. Each point represents the mean of data from two separate experiments each carried out in triplicate.

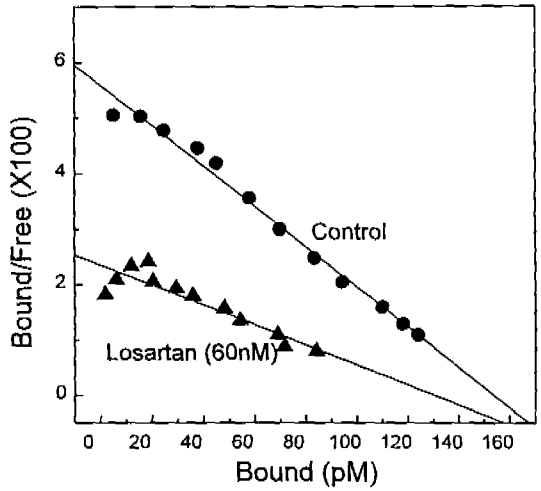

Fig. 2. Scatchard plot from binding of [^{3}H]AII in rat liver homogenates : K_d, 2.56 nM for control and 4.07 nM for Losartan; B_{max}, 352 fmol/mg for control and 336 fmol/mg for Losartan. Each point is the mean of two typical determinations in triplicate.

nts were performed with Losartan (60 nM). As shown in Fig. 2, Losartan displayed competitive kinetics as an inhibitor of [^{3}H]AII binding. It increased the K_d value without affecting B_{max} in the Scatchard plot; K_d value of rat liver membranes was 2.56 nM for vehicle and 4.07 nM for Losartan (60 nM). The B_{max} of rat liver membranes was 352 fmol/mg protein for vehicle and 336 fmol/mg protein for Losartan (60 nM).

Competition Binding Analysis in Rat Liver Homogenates

Losartan and Sarile, the nonpeptide and peptide AT_1 antagonist, caused the concentration-dependent displacement of specifically bound [^{3}H]AII in rat liver homogenates. In this preparation, these inhibitors completely displaced the specific binding of [^{3}H]AII at 10^{-5} M (Fig. 3). The rank order of potencies of the various standard materials in inhibiting the binding of [^{3}H]AII was AII>Sarile>Losartan>PD 123177 (Table I). The PD 123177, a selective AT_2 subtype antagonist did not displace the radioligand in the concentration lower than 10 μM, demonstrating that the binding site was different.

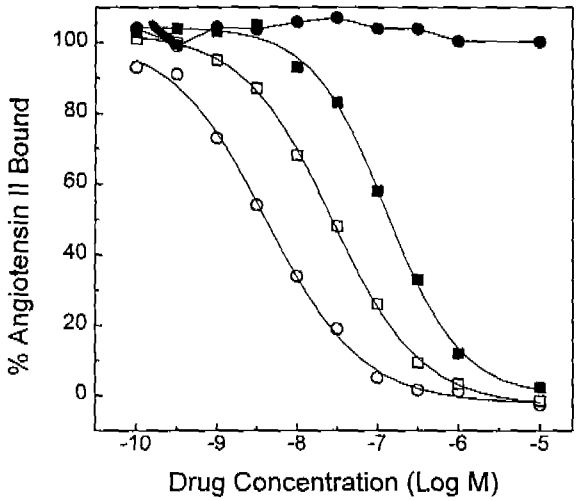

Fig. 3. Competition studies with [^{3}H]AII (3 nM) in rat liver homogenates. Data shown as mean for at least two experiments carried out in triplicate.
○=AII, □=[Sar^1, Ile^8] AII, ■=Losartan, and ●=PD 123177.

Table II. The effect of BSA on the AT_1 receptor binding assay

Compounds	IC_{50}(BSA 0.05%)	IC_{50}(BSA 0.25%)[a]	SP[b]
Losartan	4.91×10^{-8}	8.04×10^{-8}	−0.21
CV-11974	5.76×10^{-9}	9.62×10^{-8}	−1.22[c]
Angiotensin II	5.57×10^{-9}	3.18×10^{-9}	+0.24
Sarile	1.21×10^{-8}	1.35×10^{-8}	−0.05

[a]The concentration of bovine serum abumin (BSA) in assay buffer. [b]Shift parameters (SP) were calculated as the log value of (IC_{50}, BSA 0.05%)/(IC_{50}, BSA 0.25%). [c]CV-11974, the active metabolite of TCV-116 developed by Takeda Chemical Industries showed significant SP value than other compounds.

The influence of BSA on ligand-receptor binding was evaluated by the binding of [^{3}H]AII in rat liver homogenates. Table II illustrates that the inhibition of the specific binding of [^{3}H]AII by unlabeled Losartan, Sarile, or AII (10^{-10} to 10^{-5}M) was unaffected by the content of BSA. Almost identical IC_{50} values were obtained in both conditions. The shift parameters (SP) were calculated to quantify those relationships as the

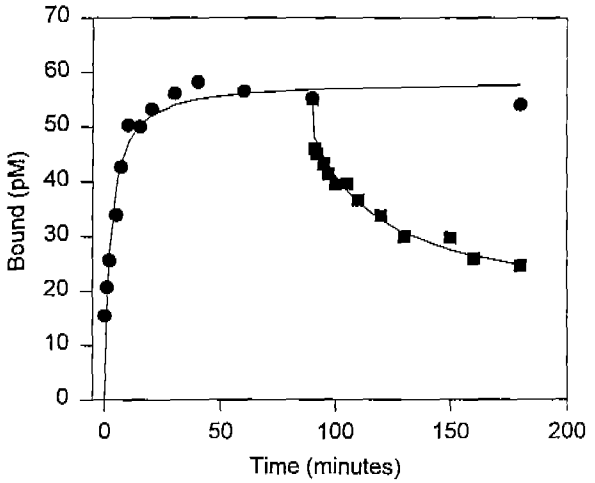

Fig. 4. Association and dissociation of [^{3}H]AII binding to rat liver homogenates incubated at 25℃. Dissocation was initiated at 90 min by the addition of unlabeled AII (1 μM). The incubation was terminated by filtration as described in methods section.

log value of the IC_{50} of 0.05% BSA to the IC_{50} of 0.25% BSA in assay buffer. The greater absolute value of SP represents more significant effect on IC_{50} value by the concentration of BSA. The CV-11974, the Takeda compound, showed the most significant effect (SP : −1.22) than other standard compounds.

Kinetic Studies of [^{3}H]AII Binding in Rat Liver

The time dependent binding of [^{3}H]AII in rat liver was measured and depicted in Fig. 4. The association was rapid and equilibrium was reached at 30 min after incubation on 25℃. At the equilibrium, a slight decrease in binding was observed but it was not significant. In the association of [^{3}H]AII, the observed association rate constants(K_{obs}) for two site were found to be 0.13 min^{-1} and 12.06 min^{-1}. The actual association rate constants were 4.10×10^7 $M^{-1}min^{-1}$ and 4.02×10^9 $M^{-1}min^{-1}$.

Unlabeled AII (1μM) dissociated the bound [^{3}H]AII, however this was not essentially reversible because about 30% of bound [^{3}H]AII was not dissociated or showed very slow dissociation. The calculated dissociation rate constant was 7.07×10^{-3} min^{-1}. This discrepancy of time kinetics might be due to the partial dissociation phenomenon. Dissociation constants ($K_d=K_{-1}/K_{+1}$) were estimated as 0.17 nM and 1.76 pM from the kinetic rate constants. The kinetically derived dissociation constants were not in reasonable agreement with the estimate of K_d obtained from equilibrium binding studies. This effect might due to the missing dissociation rate constant of undissociated site.

Discussion

The studies for the development of new nonpeptide AII receptor antagonists has greatly facilitated the characterization and subclassification of AII receptors in various tissues. While the functional role of the AT_1 subtype is becomming more clear, confusion still exists on the functional role of the AT_2 subtype and the subclasses of AT_1 subtype. Recently, Chiu *et al.* (1993) suggested that AT_{1a} and AT_{1b} should be referred to as AT_1 receptor isoforms since they can not be distinguished by pharmacological markers. However, the possibility of new findings to classify subtypes of AT_1 receptor by newly synthesized compounds still exists.

In aspect of receptor quantity, bovine adrenal is a good source for AT_1 receptor because it can be obtained easily in large quantity, however, it is difficult to separate the cortex from the medulla and thus the possible contamination can cause the undesirable heterogeneousity of the receptor preparations. AII receptor in rat adrenal cortex is the mixture of AT_1 and AT_2 with the ratio of 7 : 3 and it can be used in dual assay for AT_1 and AT_2 by adding high concentration of AT_1 or AT_2 selective antagonist to the assay system(data not shown). However, rat adrenal cortex is too small to screen a large number of compounds. That is one of the reasons to use the rat liver as AT_1 receptor source. In case of rat adrenal medulla, it associated rapidly but the equilibrium was not stable(data not shown) and so it is not satisfactory to use at reproducible screenig system for AT_2 receptor antagonists. Recently, the AT_2 receptor preparation from bovine cerebellum is being used for that purpose.

Equilibrium studies in rat liver with [^{3}H]AII revealed a single binding site of AII, but two sites were found in kinetic studies. One possibility of this phenomenon, as has been described by others (Campanile *et al.*, 1982; Crane *et al.*, 1982; Gunther, 1984), is the failure of reducing the [^{3}H]AII concentration below 50 pM, since at the concentration of [^{3}H]AII below 100 pM, the ratio of signal to noise rapidly deteriorates. The other application of the saturation experiment is to figure out the mode of action of certain antagonists, and then to divide into groups according to their mode of antagonistic action. Losartan, the nonpeptide AT_1 antagonist, showed a typical competitive antagonism to [^{3}H]AII (Fig. 2).

Another approach for grouping active compounds is the use of competition binding analysis with varying concentration of BSA in the assay mixture. In case

of CV-11974, the active metabolite of TCV-116 developed by Takeda Chemical Industries, the great shift of competition curve by the change in concentration of BSA (Table II) was due to the carboxylic acid moiety of CV-11974 (Shibouta *et al.*, 1993). The DuP 532 was also presented the significant influence on BSA on ligand-receptor binding (Chiu *et al.*, 1991). This type of grouping of compounds may help the study of receptor-ligand interaction and drug development.

Rat liver microsomal fraction had very homogeneous population of AT_1 receptor, since specifically bound [^{3}H]AII was not inhibited by PD 123177, the nonpeptide antagonist of AT_2 (Fig. 3). That can be another reason to choose the rat liver as the source of receptor for the screening of AT_1 antagonist. The AT_1 receptor binding assay using the homogeneous AT_1 receptor preparation from rat liver could be an efficient system for the screening of hundreds of newly synthesized candidate compounds of AII antagonist with reproducible results.

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