Charge heterogeneity of the AT$_1$ angiotensin II receptor subtype in the rat lung

M Montiel, J Quesada and E Jiménez
Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad de Málaga, 29080-Málaga, Spain

Abstract

In order to obtain more information on the molecular structure of the angiotensin II (Ang II) binding sites from whole rat lung membranes these were characterized by isoelectric focusing (IEF) and SDS-PAGE. Whereas a single population of Ang II receptor sites was identified ($K_d = 2.2 \pm 0.3$ nM; $B_{\text{max}} = 203.9 \pm 15.8$ fmol/mg protein) by Scatchard analysis, using IEF three Ang II binding isoforms were observed; a major band which migrated to isoelectric point (pl) 6.7, and two minor bands with pl values of 6.5 and 6.3. Specific binding of $^{125}$I-Ang II to rat lung membrane preparations was sensitive to Losartan, a non-peptide AT$_1$ receptor subtype antagonist, but was unaffected by the AT$_2$ receptor subtype antagonist CGP42112A. Immunoblotting analyses on SDS gels, using a monoclonal antibody specific to the AT$_1$ receptor, showed two immunoreactive protein species of 45 and 48 kDa. Enzymic deglycosylation using recombinant N-glycanase did not alter the molecular weight patterns of the AT$_1$ receptor subtype. The results of the present study demonstrated that the Ang II receptor population in the whole rat lung consists solely of the AT$_1$ receptor subtype and that the AT$_2$ receptor subtype is absent. In addition, the data showed the existence of charge heterogeneity of the AT$_1$ receptor subtype, and suggest that glycosylation probably does not contribute to its charge heterogeneity.

Journal of Endocrinology (1995) 147, 153–159

Introduction

Angiotensin II (Ang II) exerts a wide range of biological actions. In addition to its well-known direct effects on blood pressure and electrolyte balance, there are other renal, hepatic, endocrine and reproductive functions, and it also has specific actions in the central nervous system (Catt & Abbott 1991). Ang II is also one of a growing number of peptide hormones that have been implicated in the regulation of cellular growth (Schelling et al. 1991).

The existence of Ang II receptor subtypes has been suggested by differences in their physiological and biochemical characteristics. Two principal classes of Ang II binding sites, designated as AT$_1$ and AT$_2$ receptors, have been described (Chiu et al. 1989, Whitebread et al. 1989). Pharmacological characterization shows that the AT$_1$ receptor subtype has a high affinity for the selective non-peptide antagonist Losartan, whereas the AT$_2$ receptor subtype selectively binds CGP42112A (Whitebread et al. 1989, De Gasparo et al. 1990). The G-protein linked AT$_1$ receptor subtype is responsible for the physiologic effects of Ang II, in most tissues inducing phosphoinositide metabolism/calcium mobilization and adenylate cyclase inhibition (Bumpus et al. 1991, Bottari et al. 1992). The intracellular signalling pathway of Ang II through the AT$_2$ receptor subtype, the biological function of which is yet to be determined, may be linked to protein tyrosine phosphatase activity (Brecbler et al. 1994).

The presence and proportion of the AT$_1$ and AT$_2$ receptors vary among different tissues/organs of the same species and within the same tissue/organ of different species (Wong et al. 1992). In agreement with these findings, we have shown the presence of different isoforms of the Ang II receptor which varied in relative abundance in several rat tissues (Jiménez et al. 1991).

Widely based investigations have established the importance of the pulmonary circulation in the conversion of the decapetide Ang I to the octapeptide Ang II. Moreover, the presence of specific Ang II receptors mediating weak contraction in pulmonary arteries (Boe & Simonsson 1981), and Ang II and Ang III stimulation of diacylglycerol formation in pulmonary artery endothelial cells have also been demonstrated (Patel et al. 1991).

In order to obtain more thorough information on the molecular structure of the Ang II receptor in whole rat lung, in the present study Ang II binding sites were characterized by SDS-PAGE and immunoblotting, using a specific antibody which recognizes the AT$_1$ receptor subtype, and by isoelectric focusing (IEF). In addition, enzymic deglycosylation using recombinant N-glycanase was carried out in order to find out whether the
polysaccharide moieties play a major role in the charge heterogeneity of the Ang II receptor.

Materials and Methods

Preparation of rat lung plasma membrane

Male adult Wistar rats were housed under a schedule of 14 h light:10 h darkness and maintained on a normal laboratory diet with tap water available ad libitum. Rats were killed by cervical dislocation and lungs were quickly removed. Whole lung membrane preparations were obtained as previously described by Glossmann et al. (1974). In brief, tissue was homogenized in ice-cold 20 mmol sodium bicarbonate/l, containing 1 µg/ml of leupeptin and aprotonin, and 0-1 µg/ml of bacitracin and phenylmethysulphonyl fluoride (Sigma Chemical Co., St Louis, MO, USA). The homogenate was filtered through two layers of gauze and centrifuged at 900 g for 15 min at 4 °C. The pellet was resuspended in the same buffer and recentrifuged. The pooled supernatants were centrifuged at 30 000 g for 30 min at 4 °C and the pellet was resuspended in 50 mmol Tris—HCl/l, pH 7-4, containing 120 mmol NaCl/l, 6 mmol MgCl2/l, 1 mg BSA/ml (Sigma Chemical Co.) and protease inhibitors.

Binding studies

To investigate Ang II receptor binding characteristics, 125I-Ang II (0-1 nmol/l, specific activity 2200 Ci/mmoll; DuPont-New England Nuclear, Dreieich, Germany) was incubated with lung membrane fractions (50 µg protein) in 200 µl 50 mmol Tris—HCl/l, pH 7-4, containing 120 mmol NaCl/l, 6 mmol MgCl2/l and 1 mg BSA/ml, at 22 °C for 45 min in the presence or absence of varying concentrations of unlabelled Ang II (Sigma Chemical Co.) or selective Ang II receptor subtype antagonists (Losartan and CPG42112A; Ciba-Geigy Ltd, Basel, Switzerland). Bound and free radioactivity were separated by adding 3-0 ml ice-cold saline to the assay tube, followed by vacuum filtration through Millipore filters (Millipore Iberica, Madrid, Spain) presoaked with assay buffer. The assay tube and filter were then rinsed with three additional washes of 3-0 ml cold saline and the filters counted in a γ—counter (Diagnostic Products Corporation, Los Angeles, CA, USA). Equilibrium dissociation constant (Kd) and concentration of receptor sites (Bmax) were determined from the competition curves using the iterative curve-fitting programme Ligand (Dr P J Munson, NICHD, NIH, Bethesda, MD, USA) (Munson & Rodbard, 1980). Non-specific binding was defined as radioactivity bound in the presence of 2 µmol Ang II/l in the incubation medium.

In IEF studies, membrane samples (100–150 µg protein) were preincubated for 15 min in the presence or absence of the selective Ang II receptor subtype antagonists Losartan (5 µmol/l) or CPG42112A (2 µmol/l), or unlabelled Ang II (2 µmol/l). This was followed by incubation at room temperature for a further 30 min with 0-2 nmol 125I-Ang II/l. Bound and free 125I-Ang II were separated by centrifugation at 10 000 g for 5 min. The pellet was resuspended and washed once more with assay buffer and recentrifuged. The resultant pellet was resuspended in 70 µl Tris buffer containing 0-5% Triton X-100, incubated for 20 min at 4 °C and centrifuged at 10 000 g for 5 min. The solubilized membrane suspensions were collected and fractionated by IEF.

IEF analysis

IEF was carried out in polyacrylamide slab gels containing 12% glycerol. A pH 3-5–10-0 gradient was achieved with 0-7% ampholine pH 3-5–10-0 and 3% ampholine pH 5-0–8-0 (LKB, Bromma, Sweden). IEF was performed along the long axis using an LKB Multiphorr II system with extensive cooling (4 °C) so that gel temperature did not rise inappropriately. Gels were prefocused for 40 min at 2000 V, 20 mA and 20 W, and the runs were carried out for 4 h at 2500 V, 20 mA and 20 W using a power supply (Multidrive XI; LKB). Gels were dried and autoradiography was performed for 1 week at −70 °C using Kodak X-ray films (XAR-5; Sigma Chemical Co.). Quantitation of the bands was carried out by densitometric scanning with a Molecular Dynamics computing densitometer (Model 300A; Sunnyvale, CA, USA).

Enzymic deglycosylation

Recombinant N-glycanase (15 U/ml; Genzyme, Cambridge, MA, USA) was added to membrane protein (150 µg), in 80 µl 33 mmol Tris—HCl/l, pH 7-5, 0-1% SDS, 1-5% Triton X-100, 0-33 mmol EDTA/l, in the presence of proteinase inhibitors. A control reaction contained no enzyme. After 18 h of incubation at 37 °C the samples were subjected to SDS-gel electrophoresis and immunoblotting.

SDS-gel electrophoresis and immunoblotting

Membrane proteins were combined with an equal volume of 2 × SDS loading dye (100 mmol Tris—HCl/l, pH 6-8, 200 mmol dithiothreitol/l, 4% SDS, 0-2% bromophenol blue, 20% glycerol and 10% β-mercaptoethanol), boiled for 3 min and loaded onto a 10% SDS-polyacrylamide gel for electrophoresis (Laemmli 1970). Proteins were then transferred to Immobilon-P membranes (Millipore). These were blocked overnight in PBS containing 5% (w/v) milk protein. After washing thoroughly with PBS containing 0-1% (v/v) Tween 20 (PBS+T), membranes were incubated with 6313/G2 monoclonal antibody (1:20 (v/v) in PBS+T) for 1 h, washed with PBS+T as before, and then
incubated for a further hour with a 1:5000 dilution of alkaline phosphatase-conjugated goat anti-mouse antiserum (Promega Co., Madison, WI, USA) in PBS+T containing 1% BSA. Colour development was carried out in 20 ml 100 mmol Tris–HCl/l, pH 9.5, containing 100 mmol NaCl/l, 5 mmol MgCl$_2$/l, and 132 µl 61 mmol nitro blue tetrazolium/l and 66 µl 173 mmol 5-bromo-4-chloro-3-indolyl phosphate/l (Sigma Chemical Co.). The reaction was stopped with 200 µl 0·5 mol EDTA/l in 50 ml PBS.

**Protein determination**

Protein concentrations were determined by the method of Lowry et al. (1951) using BSA as standard.

**Results**

The saturability of specific Ang II binding to receptor in rat lung, as a function of radioligand bound in the presence of unlabelled Ang II, was determined by Scatchard analysis. This indicated a single population of Ang II receptor sites with an equilibrium dissociation constant ($K_d$) of 2·2±0·3 mmol/l (mean±s.d., n=3) and an apparent maximal Ang II receptor concentration (B$_{max}$) of 203·9±15·8 fmol/mg protein (Fig. 1).

To characterize the binding sites, $^{125}$I-Ang II binding was examined in the presence of unlabelled hormone and Ang II receptor antagonists (Fig. 2). Losartan, a potent non-peptide AT$_1$ receptor antagonist, inhibited the specific binding of $^{125}$I-radiolabelled hormone to rat lung in a concentration-dependent monophasic fashion. In contrast, CGP42112A, a selective AT$_2$ receptor antagonist, did not displace the radioligand at concentrations up to 1 µmol/l.

A representative pattern of the $^{125}$I-Ang II–receptor complex isoforms after IEF analysis is illustrated in Fig. 3. In membrane preparations from rat lung, three specific Ang II binding complexes were observed which migrated to pl 6·7, 6·5 and 6·3. Data from three separate experiments showed that these isoforms represented 74·9±6·1%, 13·2±2·0% and 11·2±1·2% of specific binding respectively. Non-specific binding of $^{125}$I-Ang II, measured in the presence of unlabelled Ang II is also demonstrated in this figure. Figure 4 demonstrates the displacement of $^{125}$I-Ang II in the presence of unlabelled Ang II from all three isoforms of Ang II receptor showing a dose-dependent manner characteristic of receptor binding. Thus, the extent of non-specific binding at pl 6·5 does not invalidate the interpretation of specific receptor subtype binding at the same pl. Further studies were carried out to confirm Ang II–receptor complex isoforms using the selective AT$_1$ receptor antagonist. The presence of Losartan (5·0 µmol/l) produced a specific inhibition of $^{125}$I-Ang II binding on all three isoforms, but this was unaffected by CGP42112A (2 µmol/l) (Fig. 3).

Our experiments were complemented by immunoblotting analyses using the monoclonal antibody 6313/G2. Two prominent immunoreactive bands with approximate molecular weights of 45 and 48 kDa were observed from fresh preparation of rat lung membranes, indicating that these immunoreactive proteins were not degradative products of the protein receptor (Fig. 5, lane 1). As shown by SDS-PAGE and immunoblotting, enzymic deglycosylation using recombinant N-glycanase did not affect the molecular weight profile (Fig. 5, lane 3).

**Discussion**

The present study was developed in order to determine which Ang II receptor isoform pattern is present in
membrane preparations from whole rat lung and to establish a relationship between these Ang II receptor isoforms and the Ang II receptor subtypes pharmacologically identified using selective Ang II receptor antagonists.

High-affinity Ang II binding sites have been demonstrated in a great variety of tissues, including the adrenal cortex and medulla, brain, kidney and noradrenergic nerve endings (Wong et al. 1992). Moreover, Ang II binding sites have also been described in pulmonary artery endothelial cells (Patel et al. 1991), suggesting that Ang II may be involved in the regulation of pulmonary perfusion and resistance (Entzeroth & Hadamovsky 1991). In agreement with these findings, a single population of Ang II receptor sites ($K_d=2.2 \pm 0.3$ nmol/l and $B_{\text{max}}=203.9 \pm 15.8$ fmol/mg protein) in pulmonary membrane preparations was observed in our experiments using Scatchard analysis.

Several pharmacological studies have identified the presence of multiple Ang II receptor forms in tissues where a single high-affinity binding site was characterized using radioligand analysis (Rogg et al. 1990, Chappell et al. 1992), and the existence of two distinct populations of Ang II receptor subtypes, AT1 and AT2, has been reported (Whitebread et al. 1989, De Gasparo et al. 1990, Sandberg et al. 1992).
Recently, using combined ligand binding and IEF analyses we demonstrated the existence of multiple isoforms of Ang II receptor in several rat tissues, not only those known to contain both Ang II receptor subtypes but also those, for example the liver, in which previously only the AT$_1$ receptor subtype was found (Jiménez et al. 1991). However, one aspect which has not been completely resolved is whether those tissues that express a single Ang II receptor subtype (i.e. AT$_1$) show similar or distinct Ang II receptor isoform patterns.

When solubilized proteins from plasma membranes of the whole rat lung were loaded on the IEF polyacrylamide gels, three Ang II–receptor complex isoforms which migrated to pl values 6-7, 6-5 and 6-3 were identified, though the pl 6-7 isoform was the most abundant. This finding was qualitatively and quantitatively different from other rat tissue profiles previously analysed (Montiel et al. 1993, Jiménez et al. 1994), clearly showing the existence of a tissue-dependent Ang II receptor isoform pattern.

Molecularly distinct Ang II receptor isoform profiles may reflect the differences in ligand binding characteristics and physicochemical properties reported in different tissues, and also between the AT$_1$ receptors in plasma membranes and nuclei from rat liver (Tang et al. 1992, Jiménez et al. 1994).

We may speculate about the physiological role of these Ang II receptor isoforms. The tissue-specific Ang II receptor isoform profile may be related to target tissue-specific effects, perhaps even activating different intracellular transduction systems. Alternatively, the existence of several Ang II receptor isoforms may be related to the processing which accompanies receptor internalization, desensitization and/or recycling.

Ang II receptor subtypes may be discriminated by their characteristic affinities for peptide and non-peptide antagonists such as CGP42112A and Losartan (Whitebread et al. 1989, De Gasparo et al. 1990). In competition-binding studies of $^{125}$I–Ang II binding to rat lung plasma membranes, the non-peptide AT$_1$ receptor antagonist Losartan blocked the total specific Ang II-binding sites and reduced $^{125}$I–Ang II binding to all three isoforms, whereas CGP42112A, a selective AT$_2$ receptor subtype antagonist, was ineffective. These findings agree with studies in which the effects of the receptor antagonist on responses to Ang II in the pulmonary vascular bed were investigated (McMahon et al. 1992) and clearly demonstrate that the AT$_2$ receptor subtype is not present in the adult rat lung, despite charge heterogeneity of the AT$_1$ receptor subtype.

To characterize the rat lung AT$_1$ receptor subtype further, our study was complemented by immunoblotting analyses using the monoclonal antibody 6313/G2. Recently, using this specific monoclonal antibody to the first extracellular domain of the AT$_1$ receptor subtype (Barker et al. 1993), a single immunoreactive band after SDS-PAGE fractionation of solubilized rat glomerulosa cells was demonstrated (Vinson et al. 1994). The 60 kDa protein represented the mature glycosylated AT$_1$ receptor subtype (Desarnaud et al. 1993). Moreover, an additional 40 kDa immunoreactive protein was detected in COS 7 cells expressing AT$_1$ receptor subtype (Barker et al. 1993), corresponding to the predicted value for unmodified AT$_1$ receptor (Sandberg et al. 1992).

In lung membrane preparations, Western blot assays showed that this antibody recognizes two proteins with approximate molecular weights of 45 and 48 kDa. These results were quite similar to those found by Paxton et al. (1993), who used polyclonal rabbit antibody and identified two immunoreactive bands of 43 and 49 kDa in rat tissues. Since there are several potential glycosylation sites in the Ang II receptor, differences in the apparent molecular weight of the Ang II receptor may be due to variations in glycosylation, and previous studies have pointed out differences in the carbohydrate content of the receptor depending on species and tissues (Carson et al. 1987).
Recently, two closely related AT₁ forms (AT₁A and AT₁B) encoded by different genes have been identified in the rat (Kakar et al. 1992). Comparison of the amino acid sequence of these two subtypes reveals a high degree of sequence homology indicating a 96% identity at the protein level. Although the differences between the two subtypes raise the intriguing possibility that these AT₁ receptor subtypes may have different functional properties and possibly different ligand-binding characteristics (Chiu et al. 1993), these variants are indistinguishable by SDS electrophoresis and hence are not identical with the two immunoreactive bands identified by Western blot analysis in the present study. Moreover, it has been demonstrated using in situ hybridization study that AT₁B subtype is absent from any tissue or cell in the lung (Gasc et al. 1994).

Since variations in carbohydrate content have been demonstrated to contribute to the physical heterogeneity of the Ang II receptor in individual target tissues, Ang II binding proteins from pulmonary membranes were treated with recombinant N-glycanase, to remove N-linked carbohydrate chains on glycoprotein. No changes in the molecular weight patterns of the immunoreactive proteins were found, suggesting no evidence for any contribution of glycosylation to the charge heterogeneity of the AT₁ receptor subtype from rat lung membranes.

Phosphorylation of the receptor molecule has been demonstrated to be an important mechanism of receptor desensitization for Ang II receptors in Xenopus oocytes (Sakuta et al. 1991). This may strongly contribute to the charge heterogeneity of Ang II receptor. Because the nucleotide sequence of the AT₁ receptor revealed a single open reading frame encoding 359 amino acids with three potential phosphorylation sites for protein kinase C (Kakar et al. 1992), further studies need to be performed to characterize the precise nature of the Ang II receptor isoforms.

Acknowledgements

The authors thank Dr Marc de Gasparo for providing CGP42112A and Losartan, and Professor Gavin P Vinson for providing 6313/G2 monoclonal antibody. This work was supported by a grant PB91-0764 from the Dirección General de Investigación Científica y Técnica, Spain.

References


Jimenez E, Vinson GP & Montiel M 1994 Angiotensin II (All)-binding sites in nuclei from rat liver: partial characterization of the mechanism of All accumulation in nuclei. Journal of Endocrinology 143 449–453.


McMahon TJ, Kaye AD, Hooi JS, Minkes RK, Nossaman BD & Kadowitz PJ 1992 Inhibitory effects of DuP 753 and E3P174 on


Received 4 October 1994
Revised manuscript received 5 April 1995
Accepted 22 May 1995