Characterization of angiotensin II receptors (binding and mRNA) in the rat thyroid gland

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ABSTRACT

In this study we showed, for the first time, the existence of a moderate density of specific angiotensin II (Ang II) binding sites ($K_d=3.9 \pm 1.7$ nM and $B_{max}=467.2 \pm 130.0$ fmol/mg protein) in plasma membrane preparations from rat thyroid gland. Reverse transcriptase/polymerase chain reactions, using primers based on the cloned AT₁ and AT₂ receptor subtypes, and pharmacological characterization, using the Ang II receptor subtype antagonists Losartan and PD 123319, revealed that these Ang II binding sites match with the AT₁ receptor subtypes. To obtain more information on the molecular structure of this Ang II receptor, immunoblotting analyses were carried out using

a polyclonal rabbit anti-AT₁ antiserum. Western analysis of fresh plasma membrane preparations from thyroid tissue showed three prominent bands of approximately 60, 45 and 40 kDa which appear to be related to different degrees of glycosylation of the receptor molecule. The functional significance of the Ang II receptors in thyroid gland is currently not known. Nevertheless, since Ang II receptors play a pivotal role in the co-ordinated actions of the renin-angiotensin system (RAS), our findings support a reciprocal regulation of thyroid function by the RAS.

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INTRODUCTION

Angiotensin II (Ang II) is a well-characterized peptide of the renin-angiotensin system (RAS) involved in cardiovascular homeostasis. In addition, Ang II stimulates cell growth and proliferation (Schelling *et al.* 1991, Steckelings *et al.* 1996), and expression of growth factors and growth-related proto-oncogenes (Naftilan *et al.* 1989). All these effects of Ang II are mediated by its binding to specific receptors. Two different Ang II receptor subtypes, AT₁ and AT₂, have been characterized by pharmacological, biochemical and molecular cloning techniques (Whitebread *et al.* 1989, Sasaki *et al.* 1991, Kambayashi *et al.* 1993).

Ang II receptor subtypes are not uniformly distributed in all somatic tissues. While some tissues express a nearly homogeneous population of the AT₁ receptor subtype (liver, lung, kidney and placenta) or AT₂ receptor subtype (pancreas and ovarian granulosa), other tissues express both receptor subtypes (adrenal, brain, uterus and heart). The physiological significance of this heterogeneous distribution of the Ang II receptor subtypes has not

yet been determined but should be important since both receptor subtypes appear to be involved in different physiological events. In general, the AT₁ receptor subtype predominates in tissues which are involved in fluid-electrolyte balance and blood pressure regulation (De Gasparo *et al.* 1995), while the AT₂ receptor subtype, which is predominantly expressed in the early stages of life (Zemel *et al.* 1990, Hermann *et al.* 1991) and re-expressed in the adult in various pathological situations (Janiak *et al.* 1992, Viswanathan & Saavedra 1992), has been suggested to play a role in tissue growth and/or differentiation (Viswanathan & Saavedra 1994, Harris & Inagami 1995, Breault *et al.* 1996).

The existence of a relationship between thyroid function and the renin-angiotensin-aldosterone system (RAAS) has been previously recognized. It is well known that thyroid hormone excess or deficiency affects several components of the RAAS, such as plasma renin activity, plasma renin concentration and plasma renin substrate and aldosterone levels (Jiménez *et al.* 1984, Montiel *et al.* 1984, Ruiz *et al.* 1987, Catanzaro 1995). Moreover, Ang II receptor densities in rat heart,

liver, kidney and adrenal gland have been found to be altered in thyroid dysfunction (Marchant *et al.* 1993, Matillas *et al.* 1993). Nevertheless, if the biologically active peptides of the RAS, such as Ang II, exert a regulating control of thyroid function, it has not yet been established. The purpose of this study was to investigate the existence of Ang II binding sites in the rat thyroid gland and to assess some of their binding and molecular properties. For the first time the gene expression and the pharmacological characterization of Ang II binding sites from thyroid gland of rats have been studied.

MATERIALS AND METHODS

Animals and preparation of plasma membranes

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*. Plasma membranes were prepared from rat thyroid glands as previously described by Glossmann *et al.* (1974). In brief, tissue was homogenized in ice-cold 20 mM sodium bicarbonate, containing 1 µg/ml leupeptin and aprotinin, and 0.1 µg/ml bacitracin and phenylmethylsulphonyl fluoride (Sigma Chemical Co., St Louis, MO, USA). The homogenate was centrifuged at 900 *g* for 15 min at 4 °C. The pellet was resuspended in the same buffer and recentrifuged. The pooled supernatants were then centrifuged at 30 000 *g* for 30 min at 4 °C and the pellet was resuspended in incubating buffer (50 mM Tris-HCl, pH 7.4, containing 120 mM NaCl, 6 mM MgCl₂, 0.1% BSA and proteinase inhibitors).

Binding assays

To investigate Ang II receptor binding characteristics, ¹²⁵I-Ang II (0.1 nM, specific activity 2000 Ci/mmol; Amersham International plc, Amersham, Bucks, UK) was incubated with thyroid gland membrane fractions (50 µg protein) in 150 µl incubating buffer at 25 °C for 45 min in the presence or absence of varying concentrations of unlabelled Ang II (Sigma Chemical Co.) or the Ang II receptor subtype antagonists Losartan (Ciba-Geigy Ltd, Basel, Switzerland) and PD 123319 (Parke Davis, Ann Arbor, MI, USA). Bound and free radioactivity was separated by adding 4.0 ml of ice-cold saline to the assay tube, followed by vacuum filtration through Millipore filters (Millipore Iberica, Madrid, Spain) presoaked with assay buffer. The assay tubes and filters were then rinsed with three additional washes of 4.0 ml cold

saline buffer and the filters counted in a γ-counter (Diagnostic Products Corporation, Los Angeles, CA, USA). Equilibrium dissociation constant (*K_d*) and concentration of receptor sites (*B_{max}*) 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 10 µM Ang II in the incubation medium.

Tissue collection and RNA extraction

Rats were killed by cervical dislocation and thyroid glands were quickly removed and frozen in liquid nitrogen. Total RNA was isolated by means of guanidine thiocyanate as previously described by Chomczynski & Sacchi (1987).

First strand cDNA synthesis and PCR assay

Total RNA (1 µg) was subjected to first strand cDNA synthesis in a 20 µl reaction volume using random primers and reverse transcriptase (Promega, Madison, WI, USA) for 45 min at 42 °C. Subsequently, these solutions were used in a PCR, in which sense oligonucleotide primer for AT₁ receptor was from the carboxyl region of the second transmembrane domain (nucleotides 250-270, 5'-TGG GCA GTC TAT ACC GCT ATG-3') and the antisense codon was from the sixth transmembrane domain (nucleotides 757-777, 5'-GAA TAT TTG GTG GGG AAC CCA-3') (Ye & Healy 1992), and for AT₂ receptor sense was from the 5' non-coding region (nucleotides 159-178, 5'-TTG CTG CCA CCA GCA GAA AC-3') and antisense from the 3' non-coding region (nucleotides 1284-1260, 5'-GTG TGG GCC TCC AAA CCA TTG CTA-3') (Nio *et al.* 1995). Denaturing, annealing, and extension reactions proceeded 40 times at 94 °C for 1 min, 55 °C for 2 min and 72 °C for 3 min for AT₁, and 94 °C for 45 s, 58 °C for 45 s and 72 °C for 1 min for AT₂. Ten microlitres of the 100 µl reaction mixture were then electrophoresed through a 1% agarose gel and stained with ethidium bromide. Contamination by genomic DNA in sample RNA was excluded by amplifying the sample RNA directly by PCR without reverse transcriptase.

SDS-gel electrophoresis and immunoblotting

Membrane proteins were combined with an equal volume of 2 × SDS loading dye (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 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). After electrophoresis, gels were equilibrated for 15 min in electroblot buffer (25 mM Tris-HCl, pH 8.3, containing 150 mM glycine), and the proteins were electrophoretically transferred to poly(vinylidene difluoride) membranes (Immobilon-P membranes, Millipore, Bedford, MA, USA) at 1 mA/cm² for 60 min using a Multiphor II electrophoresis unit (LKB, Bromma, Sweden). After transfer, the membranes were incubated for 1 h in blocking buffer (PBS containing 5% BSA). After washing thoroughly with PBS containing 0.1% (v/v) Tween-20 (PBS+T), membranes were incubated for 1 h with rabbit anti-Ang II type 1 receptor polyclonal antibody (Biogenesis, Poole, Dorset, UK) or preimmune rabbit serum diluted 1:1000 in PBS+T, washed with PBS+T as before, and then incubated for a further hour with a 1:30 000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma Chemical Co.). Colour development was carried out in 20 ml 100 mM Tris-HCl, pH 9.5, containing 100 mM NaCl, 5 mM MgCl₂ and 132 μ l 61 mM nitroblue tetrazolium and 66 μ l 173 mM 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co.). The reaction was stopped with 200 μ l 0.5 M EDTA in 50 ml PBS.

Protein determination

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

RESULTS

To examine the presence and ligand-binding characteristics of the Ang II receptor from thyroid gland, plasma membranes were incubated in the presence of ¹²⁵I-Ang II. As shown in Fig. 1, the Scatchard analysis revealed a single class of binding site with K_d and B_{max} values of 3.9 ± 1.7 nM and 467.2 ± 130.0 fmol/mg protein respectively. A Hill coefficient of 0.98 ± 0.02 ($n=3$) was indicative of a single binding site. Moreover, competition experiments were performed to confirm the pharmacological specificity of ¹²⁵I-Ang II binding in the presence of various concentrations of the specific antagonists Losartan and PD 123319. As shown in the representative experiment of Fig. 2, the AT₁ antagonist Losartan was capable of competing for these binding sites ($IC_{50}=1.9 \pm 0.8 \times 10^{-7}$ M), while PD 123319, which is representative of another series of non-peptide Ang II receptor antagonists,

FIGURE 1. Representative Scatchard plot of ¹²⁵I-Ang II binding to plasma membranes from rat thyroid gland.

FIGURE 2. Effect of increasing concentration of Ang II (●), Losartan (○) and PD 123319 (△) on specific binding of ¹²⁵I-Ang II to membranes isolated from rat thyroid gland. Each point represents the mean of triplicate determinations.

was basically inactive at concentrations lower than 10^{-6} M.

Total RNA was isolated from thyroid gland and analyzed by RT-PCR using primers based on the cloned AT₁ and AT₂ receptors to assess the presence of the mRNA encoding typical AT₁ and AT₂ receptors in thyroid gland. As a control, total RNA was isolated from rat adrenal tissue, which contains both AT₁ and AT₂ receptors. PCR analysis of isolated DNA using the primers for the AT₁ and AT₂ receptors yielded products of the expected sizes (data not shown), demonstrating that the

FIGURE 3. Agarose gel electrophoresis of RT-PCR and PCR products of rat adrenal (AG) and thyroid (TG) glands RNA with AT₁ and AT₂ receptor oligonucleotide primers. Lanes 1 and 5, RT-PCR of AT₁; lanes 2 and 6, PCR of AT₁; lanes 3 and 7, RT-PCR of AT₂; lanes 4 and 8, PCR of AT₂; lane 9, DNA markers (pBR328 DNA cleaved with BglII and HinfI).

primers amplified DNA sequences specific for the AT₁ or AT₂ receptor. As shown in Fig. 3, both AT₁ and AT₂ mRNA were detected in rat adrenal gland by RT-PCR. However, only AT₁ mRNA was detected in thyroid gland preparations. To rule out contamination of genomic DNA in our RNA preparations, we subjected RNA from tissues used for this study to PCR omitting the reverse transcriptase step; no PCR product was detected on the agarose gel after 40 cycles (Fig. 3).

Our experiments were complemented by immunoblotting analyses to characterize the molecular size of these Ang II binding sites. Three immunoreactive bands with approximate molecular masses of 60, 45 and 40 kDa were observed from fresh preparations of rat thyroid gland membranes, while a prominent band at 58 kDa was found in rat adrenal gland samples (Fig. 4).

All results are expressed as means $\pm$ S.D.

DISCUSSION

The thyroid is an endocrine gland which secretes hormones vital for metabolism and growth. In addition to the well known action of thyroid-stimulating hormone in controlling the activity of the thyroid, other humoral agents, such as growth hormone (Grunfeld *et al.* 1988), insulin and insulin-like growth factor (Santisteban *et al.* 1987), have been demonstrated to play an important regulatory role in thyroid function.

FIGURE 4. Immunoblotting of SDS gel of solubilized proteins from rat adrenal (AG) and thyroid (TG) glands. Fractionated proteins were transferred onto poly(vinylidene difluoride) membranes and Western blotted with rabbit anti-AT₁ receptor polyclonal antibody (A) or preimmune rabbit serum (B).

The results of the present study show, for the first time, the presence of specific receptors for Ang II in the rat thyroid gland, which provides evidence that this gland may also be under the direct control of the RAS.

¹²⁵I-Ang II labels a single population of binding sites in plasma membrane preparations from thyroid gland. The K_d was in good agreement with previously reported data obtained from preparations of several different tissues of the rat (Douglas 1987, Entzeroth & Hadamovsky 1991). The number of binding sites which can be labelled by the radioligand in our preparations was lower than those reported in rat liver (Booz *et al.* 1992), but higher than those reported in rat vascular smooth muscle cells (Gunther *et al.* 1980). This finding indicates that Ang II binding sites in this tissue may not be exclusively located in vascular tissue.

Ang II receptor subtypes may be distinguished by their characteristic affinities for antagonists such as PD 123319 and Losartan (Wong *et al.* 1992). In competition binding studies of ¹²⁵I-Ang II binding to rat thyroid gland plasma membranes, the non-peptide AT₁ receptor antagonist Losartan

blocked the specific Ang II binding sites, whereas PD 121339, a selective AT₂ receptor subtype antagonist, was ineffective at low concentrations. These findings demonstrate that the AT₁ receptor subtype is only present in the rat thyroid gland. Definitive evidence for the expression of the AT₁ receptor subtype in rat thyroid was obtained by PCR after reverse transcription using primers based on the cloned AT₁ and AT₂ receptors. Amplification of cDNA for AT₁ and AT₂ in both rat adrenal and thyroid gland provide good evidence that rat adrenal gland contains AT₁ and AT₂ receptor subtypes, as has been previously described (Kakar *et al.* 1992), while the AT₁ receptor subtype is only expressed in the thyroid gland.

Variations in carbohydrate content have been demonstrated to contribute to the physical heterogeneity of the Ang II receptor in individual target tissues of a single species and within the same tissue in different species. PAGE studies of solubilized AT₁ receptors have revealed bands with molecular masses ranging from 58 to 79 kDa (Catt *et al.* 1987). In order to obtain more information on the molecular structure of the Ang II receptor from thyroid gland, our study was complemented by immunoblotting analyses. Western blot assays identified three prominent bands of approximately 60, 45 and 40 kDa in fresh plasma membrane preparations from thyroid tissue, which appeared to be related to different degrees of glycosylation of the receptor molecule. Similar experiments, using plasma membranes prepared from COS-7 cells expressing the AT₁ receptor subtype, identified a 40 kDa immunoreactive protein (Barker *et al.* 1993), corresponding to the predicted value for unmodified AT₁ receptor (Sandberg *et al.* 1992). Moreover, a 60 kDa immunoreactive band has been detected in plasma membrane preparations from glomerulosa cells (Vinson *et al.* 1994), which represent the mature glycosylated AT₁ receptor subtype (Desarnaud *et al.* 1993).

In summary, the present study demonstrates the presence of type AT₁ Ang II binding sites in the rat thyroid gland.

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