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Binding of angiotensin and atrial natriuretic peptide in brain of hypertensive rats

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Atrial natriuretic peptides, produced in the mammalian cardiac atrium, are released into the general circulation and may be actively involved in the control of blood pressure and in fluid homeostasis as antagonists of the peripheral angiotensin system[1–4]. Certain cardiovascular effects of atrial natriuretic peptides may be centrally mediated, as binding sites for atrial natriuretic factor (8–33) (ANF) have been localized to the subfornical organ[5]. This circumventricular structure lacks a blood–brain barrier and is therefore accessible to circulating peptides. It contains large numbers of angiotensin II (AII) binding sites, and has been suggested as the main central site of action for circulating AII in the regulation of blood pressure and fluid metabolism[6,7]. Here we have studied binding sites for rat atrial natriuretic peptide(6–33) (rANP) and AII in the brains of spontaneously (genetic) hypertensive rats (SHR) and their normotensive controls, Wistar Kyoto (WKY) rats[8], by quantitative autoradiography[5,9–11]. Binding sites for both peptides were highly localized in the subfornical organ. The number of rANP binding sites was decreased in the subfornical organ of both young (4 weeks old) and adult (14 weeks old) SHR compared with age-matched normotensive controls. Conversely, the number of AII binding sites was higher in both young and adult SHR compared with WKY rats. Our results suggest a central role for rANP and AII in genetic hypertension; they may act as mutual antagonists in brain areas involved in control of blood pressure and fluid regulation.

rANP closely resembles rat atrial natriuretic factor(8–33) containing only two more amino acids (serine and leucine) at the N-terminus[12]. Atrial natriuretic peptides have natriuretic and hypotensive effects in normal rats[1,2] and antihypertensive actions in experimentally hypertensive rats[13] and in SHR[14]; they may lower hypertension in SHR through a direct vasodilator effect, as hypertension in this model is associated with increased peripheral resistance[15].

We have compared AII and rANP binding in brain of young (4 weeks old) and adult (14 weeks old) SHR with that in their age-matched normotensive WKY controls, to determine whether any differences observed occurred early in the development of the hypertension, or were a consequence of the chronic increase in blood pressure. Age-dependent alterations in brain neurotransmitters have been described previously in SHR[16,17].

Saturable, single-class binding sites for ^{125}I-rANP were localized in the subfornical organ (Table 1; Figs 1, 2). Nonspecific binding, determined by the addition of unlabelled rANP (Table 1, Fig. 1) or unlabelled ANF(8–33) (results not shown) was less than 30% of the total binding. Scatchard analysis, performed using consecutive sections from individual brains, demonstrated decreased maximum binding capacity (B_{max}) in the subfornical organ of both young and adult SHR compared with age-matched WKY rats (Table 1, Fig. 2). Also, the affinity constant for binding (K_a) was lower in the subfornical organ of adult SHR than in that of WKY rats (Table 1).

The choroid plexus also showed a high concentration of ^{125}I-rANP binding sites. Values of B_{max} were lower in choroid plexus of SHR than in age-matched WKY rats (74 ± 13 and 21 ± 3 fmol per mg protein for 4-week-old WKY rats and SHR, respectively: 120 ± 14 and 61 ± 10 fmol per mg protein for 14-week-old WKY rats and SHR, respectively; $P < 0.05$) but the K_a values showed no difference at either age between the two strains (9.1 ± 1.9 and 11.3 ± 2.2 × 10^9 M^{-1} for 4-week-old WKY rats and SHR, and 7.6 ± 2.0 and 6.3 ± 0.8 × 10^9 M^{-1} for 14-week-old WKY rats and SHR, respectively). No differences in concentration or affinity of rANP binding sites between WKY rats and SHR were found in another forebrain area rich in rANP sites, the olfactory bulb (not shown).

Differences in the numbers of AII binding sites in SHR compared with WKY rats were evident in the subfornical organ of both young and adult animals, but these differences were the

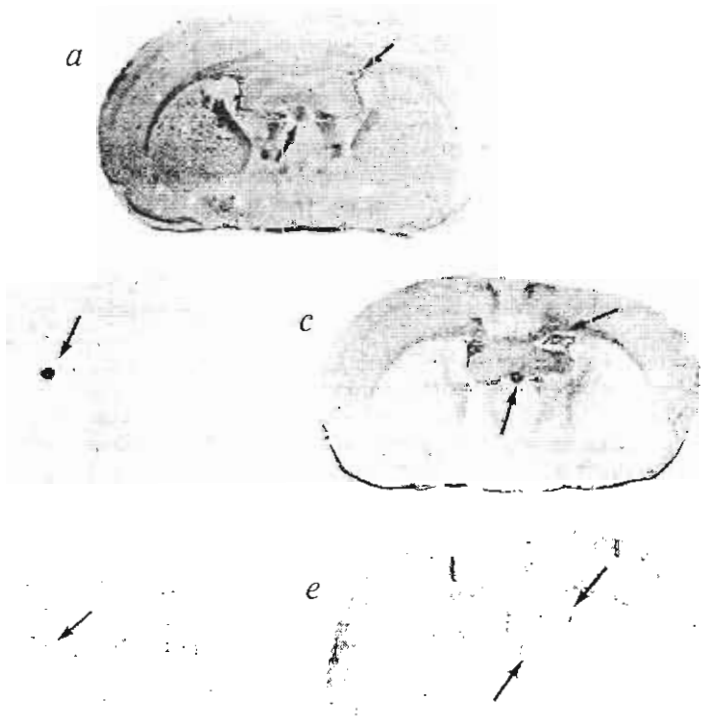

Fig. 1 Autoradiographic images of ^{125}I-rANP and ^{125}I-[Sar^1]AII binding in rat brain at the subfornical organ level. *a*, Section stained with Luxol fast blue. *b*, Section incubated with 1 nM ^{125}I-[Sar^1]AII and exposed to ^{3}H-Ultrofilm for 2 days. *c*, Section incubated as in *b*, with the addition of 1 μM unlabelled AII. *d*, Section incubated with 400 pM ^{125}I-rANP and exposed to ^{3}H-Ultrofilm for 3 days. *e*, Section incubated as in *d*, with the addition of 1 μM unlabelled rANP. All sections are from a 14-week-old WKY rat. Arrows indicate the subfornical organ (*a–e*) and the choroid plexus (*a*, *d* and *e*).

Table 1 rANP and AII binding sites in subfornical organ of spontaneously hypertensive rats compared with normotensive controls (WKY)

Peptide	Age (weeks)	Binding capacity, B_{max} (fmol per mg protein) WK	SHR	Affininity constant, K_a($\times 10^9$ M^{-1}) WK	SHR
Atrial natriuretic peptide	4	85 ± 12	38 ± 4*	8.52 ± 1.30	6.92 ± 1.53
	14	124 ± 11	73 ± 10*	11.10 ± 1.67	4.25 ± 0.55*
Angiotensin II	4	444 ± 85	735 ± 91*	0.69 ± 0.10	0.32 ± 0.06*
	14	848 ± 136	1,390 ± 64*	1.90 ± 0.36	0.89 ± 0.05*

Groups of six 4-week-old and 14-week-old male SHR and WKY rats (Taconic Farms, Germantown, New York) were housed at a constant temperature with illumination from 06.00 to 18.00 h and given free access to food and water. Blood pressures, measured the day before killing of the animals by an indirect tail-cuff method using a Programmed Sphingomanometer (Narco Biosystems, Inc., Houston, Texas), were 100 ± 8 and 125 ± 10 mm Hg for 4-week-old WKY rats and SHR, respectively, and 120 ± 10 and 184 ± 12 mm Hg for 14-week-old WKY rats and SHR, respectively ($P < 0.01$). The rats were killed by decapitation between 09.00 and 11.00 h and their brains were immediately removed and frozen by immersion in isopentane (−30 °C). Within 24 h of killing, tissue sections (16 μm) were cut in a cryostat at −14 °C, thaw-mounted onto subbed glass slides, and placed under vacuum at 4 °C until incubation. rANP binding sites were labelled *in vitro* by incubation with ^{125}I-labelled 3-iodotyrosyl^{28} rANP (specific activity 1,750 Ci mmol^{-1}; Amersham). Tissue sections were preincubated for 15 min at 20 °C in 50 mM Tris-HCl buffer, *p*H 7.4, then incubated at room temperature for 60 min in 50 mM Tris-HCl buffer, *p*H 7.4, containing 100 mM NaCl, 5 mM $MgCl_2$, 0.5% bovine serum albumin (BSA), 40 μg ml^{-1} bacitracin, 4 μg ml^{-1} leupeptin, 2 μg ml^{-1} chymostatin, 0.5 μg ml^{-1} phenylmethylsulphonyl fluoride (PMSF) and ^{125}I-rANP in concentrations ranging from 10 to 400 pM. Nonspecific binding was determined in consecutive sections in the presence of 25 nM to 1 μM of unlabelled rANP (rat atrial peptide, 28 amino acids; Peninsula Laboratories, Inc., Belmont, California). After incubation, the slides were washed three times (for 2 min each) in ice-cold Tris-HCl buffer at 4 °C and dried under a cold stream of air. To determine AII binding, tissue sections were preincubated for 15 min in 10 mM sodium phosphate buffer (*p*H 7.4) containing 120 mM NaCl, 5 mM Na_2EDTA, 0.1 mM bacitracin (Sigma) and 0.2% BSA. The tissue sections were then incubated for 60 min in fresh buffer, with ^{125}I-[Sar^1]AII (specific activity 1,666 Ci mmol^{-1}) in concentrations from 80 pM to 5 nM. Nonspecific binding was determined in alternate sections incubated in the presence of 80 nM to 5.0 μM unlabelled AII (Sigma). After incubation, the slides were washed four times in ice-cold 50 mM Tris-HCl buffer and dried under a stream of air. Autoradiographic images were produced by placing the slides in X-ray cassettes and exposing them to ^{3}H-Ultrofilm for 4 days. rANP and AII binding sites were quantified by autoradiography with ^{3}H-Ultrofilm (LKB), followed by computerized microdensitometry and comparison with ^{125}I standards[10,11]. Scatchard plots were calculated by linear regression. Values are the mean (±s.e.m.). Statistical differences between groups were analysed using the Student's *t*-test.

* Significant difference compared with WKY rats ($P < 0.05$).

reverse of those found for rANP binding sites (Table 1, Fig. 2). SHR exhibited significantly more binding sites for AII than normotensive rats at both ages. Affinity constants, however, were lower in young and adult SHR than in WKY rats (Table 1, Fig. 2). These differences were specific to the subfornical organ, as they were not evident in another circumventricular structure, the area postrema[18].

The subfornical organ has a crucial role in the central effects of circulating AII in the regulation of blood pressure and fluid homeostasis, including regulation of vasopressin secretion[19]. Our results suggest that this structure may represent the site for central actions of atrial natriuretic peptides also[5]. The subfornical organ sends projections to the hypothalamus, specifically to the anteroventral/third ventricle (AV3V) region, an area critical for the development and maintenance of experimental hypertension as well as fluid and electrolyte balance[20]. This area contains the largest accumulation of ANF-positive cells in the brain[21] and large numbers of AII-positive nerve terminals[22] and receptors[9-11]. AII nerve cells and terminals[22] and the angiotensin-converting enzyme kininase II are also concentrated in the subfornical organ[23,24]. Thus, the subfornical organ and its hypothalamic projections may represent the site of interactions between circulating and brain neuropeptides such as atrial natriuretic peptides and AII.

Several lines of evidence link both AII and atrial natriuretic peptides with blood pressure regulation. A generalized decrease in the activity of the peripheral AII system[25-28] and increased activity of the central AII system occur in SHR[29-31]; these animals also have a decreased cardiac ANF content, and increased blood levels of the peptide[1,32]. Both AII and atrial natriuretic peptides play a part in the secretion of vasopressin, a peptide whose levels are increased in spontaneous hypertension[33]. Further, AII stimulates vasopressin release via central mechanisms[34]. Administration of AII increases blood pressure [6,20] while injection of ANF has a hypotensive effect[14,35,36]. This evidence, when considered in conjunction with the present results, suggests that, in comparison with WKY rats, the increased number of AII binding sites and the decreased number of rANP binding sites in the subfornical organ may both be related to increased vasopressin activity in SHR[33].

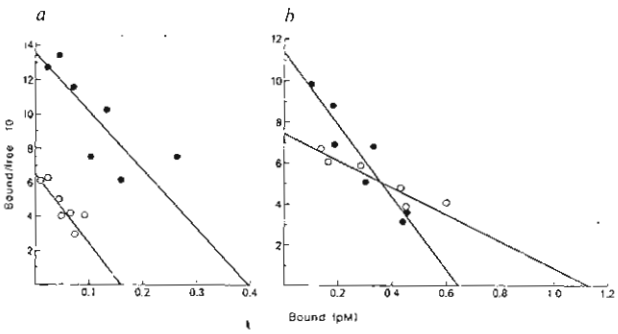

Fig. 2 Scatchard analysis of specific ^{125}I-rANP (*a*) and ^{125}I-[Sar^1]AII (*b*) binding in subfornical organ of 4-week-old SHR (○) and WKY rats (●). Consecutive tissue sections from individual rats were incubated with different concentrations of ^{125}I-labelled peptides as described in Table 1. The data represent the results of typical experiments which were replicated six to seven times per group (see Table 1). Values of K_a, B_{max} and correlation coefficient, *r*, were as follows. [Sar^1]AII binding in WKY rats: $K_a = 0.88 \times 10^9$ M^{-1}, $B_{max} = 355$ fmol per mg protein, $r = 0.828$; in SHR: $K_a = 0.32 \times 10^9$ M^{-1}, $B_{max} = 702$ fmol per mg protein, $r = 0.940$. *r*-ANP binding in WKY rats: $K_a = 3.33 \times 10^9$ M^{-1}, $B_{max} = 113$ fmol per mg protein, $r = 0.742$; in SHR: $K_a = 4.02 \times 10^9$ M^{-1}, $B_{max} = 46$ fmol per mg protein, $r = 0.900$.

Independently, an increased number of AII binding sites in the subfornical organ further substantiates the idea of an overactive central AII system in spontaneous hypertension[29-31,37], while the decreased number of rANP binding sites suggests that decreased central atrial natriuretic peptide activity may be associated with spontaneous hypertension. As the changes in AII and rANP binding are already present in early hypertensive, young SHR, they are probably not the result of chronic alterations in blood pressure. In the periphery, atrial natriuretic peptides could act as physiological antagonists of the renin-angiotensin system, as they antagonize the increased aldosterone production and vasoconstriction produced by AII[1-3]. Our results indicate that this antagonism could exist in selected brain areas also.

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