BINDING SITES FOR ATRIAL NATRIURETIC PEPTIDE IN HIGH CONCENTRATIONS IN HUMAN OVARY

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ABSTRACT

As one approach to examining the molecular functions of atrial natriuretic peptide (ANP), we examined the binding capacities of four human ovaries, one with a developing follicle of 7 mm (a) in diameter, two preovulatory follicles (small one of 12 mm (b) and large one of 18 mm (c) in diameter just before ovulation) and one (d) at menopause. The number of ANP binding sites of the ovary with a Graafian follicle (c) was far greater than those previously observed in various tissues. Four ovaries showed a drastic difference in concentrations of ANP binding sites. The binding capacity of membrane fractions from the ovary with a Graafian follicle just before ovulation (c) was 18 times greater than that of the ovary at menopause (d), 7.2 times that of the developing follicle (a), and 3.8 times that of the ovary with a preovulatory follicle (b). However, the dissociation constant (K_d) values showed no major difference at any stage among three ovaries except ovary at menopause. These findings suggest that ANP is involved in events related to the reproductive process.

Atrial natriuretic peptides (ANPs) modulate blood-pressure homeostasis, aldosterone production, and extracellular fluid volume (6, 7, 21). ANPs are known to affect various regions of the brain, the adrenal gland, the kidney, and the vascular system by combining with specific binding sites (8, 13, 16, 19, 23). ANPs have binding sites also in the lung (17), the liver (29), the placenta (24), and the ciliary bodies (2). However, the effects of ANPs in these tissues are still unclear. Recently, a high concentration of extrarenal prorenin was shown in the human ovarian follicular fluid (hFF) (9), and renin messenger RNA was found by us in the rat ovary (11). Moreover, the presence of angiotensin II (A II)-like immunoreactivity in the hFF (5) and the presence of A II receptors in the rat ovary (27) were reported. These results suggest that the renin-angiotensin system plays an important

role in events related to the reproductive process, and also that ANPs are involved in the process of folliculogenesis, either as physiological antagonists of the renin-angiotensin system or as regulators of sodium and water transport (4, 7, 12).

To determine whether any difference occurred in the process of follicular maturation, we have compared the ANP binding sites in four human ovaries, one with a developing follicle (7 mm in diameter (a)), two with preovulatory follicles (small one of 12 mm (b) and large one of 18 mm (c) in diameter just before ovulation), and one (d) at menopause.

MATERIALS AND METHODS

Tissues and Chemicals

Ovarian follicles (7 mm, 12 mm, and 18 mm in diameter) and ovary at menopause were

Fig. 1 Ligand-binding properties of membranes from the human ovaries. A: Saturation curve of ^{125}I -rANP binding to membrane particles from the human ovaries. B: Scatchard analysis of ^{125}I -rANP-binding. C: ANP binding sites in human ovaries. a, ovary with a developing follicle (7 mm in diameter); b, ovary with a preovulatory follicle (12 mm in diameter); c, ovary with a preovulatory follicle (18 mm in diameter) just before ovulation; d, ovary at menopause. Each point represents the mean of triplicate incubations. Values of K_d , $B_{\max}$, and correlation coefficient (r) were as follows. ANP bindings: a, $K_d=8.7 \times 10^{-9}$ M, $B_{\max}=2.56$ pmol per mg protein, $r=0.87$; b, $K_d=8.12 \times 10^{-9}$ M, $B_{\max}=4.92$ pmol per mg protein, $r=0.91$; c, $K_d=8.54 \times 10^{-9}$ M, $B_{\max}=18.46$ pmol per mg protein, $r=0.98$; d, $K_d=2.10 \times 10^{-9}$ M, $B_{\max}=1.02$ pmol per mg protein, $r=0.80$.

obtained from patients with uterine myoma or carcinoma *in situ* during abdominal total hysterectomy with tubo-oophorectomy. Kidney was obtained from a patient with renal cell carcinoma and the normal part was used. ^{125}I -rat ANP (^{125}I -rANP, 2,087 Ci/mmol) was purchased from Amersham, U.K., and synthetic rANP was from Peptide Institute, Osaka, Japan.

Membrane Preparations and Radioligand Assay of ANP-binding Site

The tissue was minced and homogenized in 50 mM Tris-HCl buffer (pH 7.1) containing 5 mM EDTA, 2 $\mu\text{g}/\text{ml}$ leupeptin, and 2 $\mu\text{g}/\text{ml}$ antipain by 5.5 strokes in a polytron PT 10/35 homogenizer. The homogenate was centri-

Fig. 2 Specificities of ^{125}I -rANP cross-linking to membranes of human ovary with a Graafian follicle just before ovulation (18 mm in diameter) and with the human kidney cortex. Human ovarian membranes (0.05 mg) (lanes, 1, 2, 5, and 6) and human kidney cortex membranes (0.05 mg) (lanes, 3, 4, 7, and 8) were incubated for 30 min at 22°C with 200,000 cpm of ^{125}I -rANP. Incubation of ^{125}I -rANP with membranes was performed in the absence (lanes, 1, 3, 5, and 7) or presence of $1.8\ \mu\text{M}$ unlabeled rANP (lanes 2, 4, 6, and 8).

fused at $10,000\ g$ for 10 min at 4°C and the supernatant was centrifuged at $105,000\ g$ for 40 min at 4°C . The pellet was suspended in 10 mM phosphate-buffered saline (PBS, 10 mM phosphate, pH 7.4, containing 120 mM NaCl and 10 mM EDTA), homogenized, and recentrifuged at $105,000\ g$ for 40 min at 4°C . Membranes were stored at -70°C until use. Membrane suspension was diluted to 50–500-fold for binding assay with PBS containing $20\ \mu\text{g}/\text{ml}$ leupeptin and $20\ \mu\text{g}/\text{ml}$ antipain. Aliquots ($180\ \mu\text{l}$ each) of diluted membrane suspensions were incubated with 2×10^4 cpm of ^{125}I -rANP (0.01 – $0.02\ \text{nM}$) in a total volume of $200\ \mu\text{l}$ containing $10\ \mu\text{l}$ of cold rANP of various concentrations. After incubation for 30 min at 22°C , 1 ml of cold PBS containing 0.1% bovine serum albumin (BSA) was added and filtered with Whatman GF/F filter. Filters were washed twice with the same buffer and radioactivity retained in the filter was measured in a Beckman gamma-counter.

Affinity Labeling, Electrophoresis, and Autoradiography

^{125}I -rANP (2×10^5 cpm) of ^{125}I -rANP was allowed to bind to membranes for 30 min at 22°C . Replicate incubations were made with the addition of $1.8\ \mu\text{M}$ unlabeled rANP to distinguish specific from nonspecific labeling. After washing the membranes twice by centrifugation, the bound ^{125}I -rANP was covalently cross-linked by adding 0.5 mM disuccinimidyl suberate (DSS, Pierce, U.S.A.). Following a 10 min incubation at 22°C , the cross-linking reaction was terminated by adding ammonium acetate to a final concentration of 200 mM, and membranes were recovered and washed by centrifugation at $100,000\ g$ for 10 min. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (4) on 3–13% gradient slab gels. For reduction of samples, 5% 2-mercaptoethanol was used. Radioactivity was detected by autoradiography using Fuji X-ray film; exposure was at -70°C for 36 h.

RESULTS

To examine the affinities and binding capacities of ANP receptors from four ovaries, we performed the Scatchard analysis with labeled ANP. The saturable, high-affinity binding was seen in the ovary (Fig. 1A) and specific binding reached equilibrium between 30 and 60 min at 22°C . The dissociation constant (K_d) showed no major differences at any stage among three ovaries (a, b and c) except at menopause (d), revealing a single class of binding sites with dissociation constants (K_d) of $8.70 \times 10^{-9}\ \text{M}$ for a, $8.12 \times 10^{-9}\ \text{M}$ for b, $8.54 \times 10^{-9}\ \text{M}$ for c, and $2.10 \times 10^{-9}\ \text{M}$ for d (Fig. 1B and Table 1). However, these ovaries showed the marked differences in concentrations of ANP binding sites. The binding capacities differed significantly in these ovaries with values of $2.56\ \text{pmol} \cdot \text{mg}^{-1}$ for a, $4.92\ \text{pmol} \cdot \text{mg}^{-1}$ for b, $18.46\ \text{pmol} \cdot \text{mg}^{-1}$ for c, and $1.02\ \text{pmol} \cdot \text{mg}^{-1}$ for d (Fig. 1B and Table 1). The binding capacity of the membrane fractions from the ovary with a Graafian follicle just before ovulation (c) was 7.2 times greater than that of the ovary with a developing follicle (a), 3.8 times that of the ovary with

Table 1 ANP Binding Sites in Various Membrane Preparations

Species	Tissues	Binding capacity $B_{\max}$ (pmol per mg protein)	Affinity constant K_d (nM)
Human	Ovary a	2.56	8.70
	b	4.92	8.12
	c	18.46	8.54
	d	1.02	2.10
Human	Kidney (cortex)	0.88	3.44
Pig	Kidney (cortex)	0.26	4.11
Pig	Lung	0.59	2.08
Pig	Ovary	15.17	1.48
Pig	Uterus	4.60	12.35
Pig	Testis	3.29	4.91

a: Ovary with a developing follicle (7 mm in diameter); b: Ovary with a preovulatory follicle (12 mm in diameter); c: Ovary with a large, preovulatory follicle (18 mm in diameter); d: Ovary at menopause. Porcine tissues were obtained from a local slaughterhouse. Porcine ovary, testis, and uterus were removed from the adult hogs. Membranes were prepared as described in Materials and Methods.

a preovulatory follicle (b), 18 times that of the ovary at menopause, and 21 times greater than that observed in human kidney cortex which has been known as a target tissue of ANP (Fig. 1C).

Using affinity labeling procedures, we found that radioactivity for ^{125}I -rANP was specifically detected in the bands of 130,000 and 66,000 daltons (Fig. 2) in the absence of 2-mercaptoethanol. In the presence of the reducing agent, the 130,000 dalton species migrated in the gel to the 66,000 dalton position (Fig. 2). These molecules are similar to those reported for other target tissues (3, 10, 22, 25, 26, 28). With the exposure time identical for an ovarian sample, the same amount of human kidney sample yielded extremely weak bands at the 130,000 and 66,000 dalton positions (Fig. 2).

Difference in the number of ANP binding sites in the adult female hog was also great in the ovary (Table 1). In the ovary ANP binding sites were more abundant than in other tissues reported to be rich in ANP binding sites.

DISCUSSION

The present study demonstrated that the number of binding sites for ANP in the ovary is greater than those previously observed in various tissues, and suggested that ANP is

involved in the regulation of ovarian function.

In concert with gonadotropin and steroid changes, ovarian tissues undergo regular cyclic changes with follicular growth, follicular maturation, ovulation, and formation, followed by regression, of the corpus luteum. Therefore, in the ovary, the circulating or locally generated ANP may have a crucial role in the regulation of follicular fluid volume, steroidogenesis, or other peptide production. Our results suggest that the ovary may represent one of the sites for central actions of ANPs. In the process of the follicle development, ANP may play a critical role in both follicular growth and formation of ovarian follicular fluid.

Recent reports suggest the possibility that ANP has a general effect on steroidogenesis. Bex and Corbin (1) have demonstrated that the ANP can produce a dose-related increase in testosterone secretion by mouse interstitial cells, while Pandey *et al.* (18) have demonstrated that ANP markedly suppresses gonadotropin-stimulated progesterone secretion in murine Leydig tumor cells. Therefore, ANP may also be involved in the steroidogenesis in the ovary.

As previously discussed (20), ANP may play a role in the secretion of vasopressin in the ovary. Although the functions of vasopressin in this tissue are not clear, a marked difference

in the ovarian content of immunoreactive arginine-vasopressin between immature and adult rats have been observed (15). This suggests the possible effects of gonadotropins on ovarian vasopressin levels. Lim *et al.* (15) demonstrated that sequential treatment with pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) significantly lowered ovarian immunoreactive arginine-vasopressin in both Long-Evans rats and homozygous Brattleboro rats. These results are consistent with the stimulation of the production of ANP binding sites by gonadotropins. Since ANP acts as a physiological antagonist of the renin-angiotensin system, it may both suppress the increased vasopressin production and antagonize fluid regulation by angiotensin II.

These findings suggest that ANP plays an important role in events related to the development of ovarian follicle, and further to the reproductive process. These possibilities highlight the need to explore the role that ovarian ANP plays.

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