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Downregulation of natriuretic peptide system and increased steroidogenesis in rat polycystic ovary☆

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ABSTRACT

Atrial natriuretic peptide (ANP) is known to regulate ovarian functions, such as follicular growth and steroid hormone production. The aim of the present study was to investigate the natriuretic peptide system in a rat model of chronic anovulation, the rat polycystic ovary. Adult female Wistar rats received a single subcutaneous injection of 2 mg estradiol valerate to induce polycystic ovaries, while the control group received vehicle injection. Two months later, their ovaries were quickly removed and analyzed. Polycystic ovaries exhibited marked elevation of testosterone and estradiol levels compared to control ovaries. The levels of ANP and the expression of ANP mRNA were highly reduced in the polycystic ovaries compared to controls. By immunohistochemistry, polycystic ovaries showed weaker ANP staining in stroma, theca cells and oocytes compared to controls. Polycystic ovaries also had increased activity of neutral endopeptidase, the main proteolytic enzyme that degrades natriuretic peptides. ANP receptor C mRNA was reduced and ANP binding to this receptor was absent in polycystic ovaries. Collectively, these results indicate a downregulation of the natriuretic peptide system in rat polycystic ovary, an established experimental model of anovulation with high ovarian testosterone and estradiol levels. Together with previous evidence demonstrating that ANP inhibits ovarian steroidogenesis, these findings suggest that low ovarian ANP levels may contribute to the abnormal steroid hormone balance in polycystic ovaries.

1. Introduction

Atrial natriuretic peptide (ANP), mainly secreted by cardiomyocytes, is well known to regulate body fluid volume and blood pressure homeostasis [27]. In addition to cardiomyocytes, several organs and tissues, including ovaries [12], produce ANP. ANP exerts its biological effects mainly through natriuretic peptide receptor A (NPR-A), which is coupled to guanylyl cyclase (GC) and generates cGMP. ANP may also bind to natriuretic peptide receptor C (NPR-C), initially described as a clearance receptor [20]. In addition to NPR-C, neutral endopeptidase (NEP), a cell surface metallopeptidase [9], also degrades ANP.

Ovarian theca and granulosa cells produce steroid hormones in response to the endocrine stimuli of luteinizing hormone and follicle-stimulating hormone, both from pituitary origin. However, multiple paracrine and autocrine factors, such as peptides and growth factors, also play a role in ovarian steroidogenesis [7,8,13,22]. Among these local factors, the natriuretic peptide system can be highlighted. There is growing body of evidence that ANP, brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP), in addition to their specific receptors, are expressed in the mammalian ovaries with specific regulation during the estrous cycle [11,14,15,25]. ANP regulates ovarian follicular growth and inhibits estradiol and progesterone release by granulosa cells, indicating its involvement in ovarian physiology [11,16,31,32,36,37].

Despite all the evidence that ANP is an important modulator of follicular development and steroidogenesis, the ovarian expression of this peptide has not been previously evaluated in pathological conditions. We have recently reported that women with polycystic ovary syndrome (PCOS) have low plasma levels of ANP [18] and we reasoned that the cause might be a reduced ovarian ANP release. Thus, we sought to investigate the expression of ANP and its receptors in a well-established model of chronic ovarian dysfunction

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and anovulation that recapitulates several features of PCOS, the rat polycystic ovary [2,3,5,21,29,30].

2. Materials and methods

2.1. Animals

Adult Wistar rats weighing 200–220 g were obtained from CEBIO (Centro de Bioterismo of the Federal University of Minas Gerais, Belo Horizonte, MG, Brazil) and were cared for according to the international guidelines for animal care. The experimental protocol was approved by the Ethics Committee for Animal Experimentation of the Federal University of Minas Gerais. The animals (5–6 per cage) were maintained under controlled light and temperature conditions (lights on from 05:00 to 19:00, 23 ± 3 °C) and had free access to tap water and a standard rat chow (Nuvital Nutrientes Ltda, Colombo, PR, Brazil).

2.2. Hormonal treatment

Estrous cycles were monitored by daily vaginal smears, and only animals exhibiting at least three regular cycles were selected. Following previous studies [2,3,5,21,29,30], polycystic ovaries were induced by a single subcutaneous injection of 2 mg estradiol valerate (EV) (Sigma–Aldrich Corporation, St. Louis, MO, USA) diluted in corn oil (0.2 ml/rat, $n = 34$). Control animals ($n = 33$) received a single injection of 0.2 ml of corn oil. The estrous cycle was monitored daily by vaginal cytology smears for at least two weeks to select the cycling rats to be sacrificed in the estrus phase, since the experimental group was acyclic and exhibited a constant estrus pattern. After 60 days, when the ovarian cysts become established [2], the rats were quickly decapitated to avoid any interference of anesthetic and the ovaries were removed, weighed, frozen in liquid nitrogen and maintained at −80 °C. Both ovaries from each animal were pooled for all analyzes, and each analytical procedure (steroids and ANP measurements, NEP activity, immunohistochemistry, autoradiography and real-time PCR) was conducted in ovaries from different animals (5–6 per experimental group).

2.3. Radioimmunoassay (RIA)

The ovaries were homogenized in methanol and maintained at 4 °C for 24 h. After centrifugation ($1500 \times g$/15 min), the supernatant was transferred to other tubes and evaporated at room temperature for 2–3 days. After extraction with ethyl ether, samples were evaporated and resuspended in phosphate buffer (pH 7.6). Ovarian testosterone and estradiol were measured using commercial kits (Adaltis, Rome, Italy), according to the manufacturer's instructions. All samples were measured in duplicate.

To quantify ovarian ANP levels, the ovaries were homogenized at 4 °C in 0.1 N acetic acid containing protease inhibitors: 10^{-5} M phenylmethylsulfonylfluoride, 0.5×10^{-5} M Pepstatin-A, 10^{-5} M EDTA (all purchased from Sigma–Aldrich Corporation, St. Louis, MO, USA). ANP from the supernatant was extracted by Sep Pak C18 columns, dried in Speed-Vac and stored at −80 °C until measurement by RIA. The samples were then reconstituted in buffer pH 7.4 (0.1 M sodium phosphate, 0.14 M NaCl, 0.1% BSA, 0.01% sodium azide, 0.1% Triton X-100), and the RIA for ANP was performed as previously described [10]. Briefly, the peptide was labeled with ^{125}I using the cloramine-T method and purified by HPLC. Rabbit polyclonal antibody (kindly donated by Dr. Jolanta Gutkowska, Université de Montreal, Montreal, CA) was used at 1:30,000, followed by goat anti-rabbit gamma globulin (1:50) and precipitation with polyethylene glycol. The assay linear range is 3.9–3900 pg/ml [10]. All samples were measured in duplicate in the same assay, and the intra-assay coefficient of variation was <10%. The results are reported as pg/mg of ovarian tissue.

2.4. NEP activity

To determine the activity of NEP, which is a main proteolytic enzyme that degrades natriuretic peptides, the ovaries were homogenized at 4 °C in 0.32 M saccharose solution/50 mM sodium borate (pH 7.4) and centrifuged ($800 \times g$/10 min/4 °C). NEP activity was determined following previous descriptions [23] using as fluorogenic substrate 1 μM Abz-Darg-Arg-Gly-Leu-EDDnp, whose cleavage by NEP releases Abz, a fluorescent marker. The substrate was added in 50 mM Tris buffer pH 8.0 at 37 °C. After 1 min of stabilization, the sample was added to the substrate solution, and the activity was read for 120 s at 420 nm emission and 320 nm excitation wavelengths by spectrofluorometry (F-2000 Fluorescence Spectrophotometer – Hitachi, Japan). Assay specificity was tested by use of Thiorphan (50 μM), which leads to the complete inhibition of NEP activity of the sample. The ovarian protein concentrations were measured by Lowry method [19]. NEP activity was reported as μM/min/mg protein.

2.5. Immunohistochemistry

The rats received an intraperitoneal injection of 5000 IU/kg heparin sulfate (Liquemine, Roche, Rio de Janeiro, Brazil) and were anesthetized with 30 mg/kg sodium pentobarbital. After laparotomy and thoracotomy, animals were perfused through the left ventricle with 0.05 M PBS containing the following protease inhibitors: 10^{-5} M phenylmethylsulfonylfluoride, 0.5×10^{-5} M Pepstatin-A, and 10^{-5} M EDTA (Sigma–Aldrich). After perfusion with 4% paraformaldehyde, the ovaries were removed and immerged in 4% paraformaldehyde and kept for 2 h at 4 °C. The ovaries were then fixed in Bouin's solution for 4 h at room temperature and transferred to 70% alcohol until free from the yellowish coloration conferred by the fixative. Finally, the ovaries were embedded in paraffin, sectioned at 4 μm, and mounted on gelatinized slides.

Immunohistochemistry was performed by the avidin–biotin–peroxidase method using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA) as previously described [28]. Ovarian sections were dewaxed in xylene, rehydrated in graded alcohols and washed in distilled water. The sections were incubated in methanol 1% hydrogen peroxide solution for 30 min, followed by normal goat serum for 30 min, to block endogenous peroxidase and nonspecific binding, respectively. The slides were incubated overnight (16–18 h; 4 °C) with rabbit polyclonal antibody anti-ANP (1:1000) (Peninsula-Bachem, Torrance, CA, USA) in PBS containing 1% bovine serum albumin (BSA; Sigma). The sections were then rinsed in PBS and incubated for 30 min with biotinylated anti-rabbit IgG (1:200). Subsequently, the sections were washed with PBS and incubated with the avidin-biotin complex (1:200) for 1 h. The immunostaining was visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma–Aldrich Corp.) and counterstained with hematoxylin. Negative controls were obtained by incubation of the slides with 1% PBS/BSA instead of the primary antibody. An additional negative control was performed by preadsorption of the primary antibody with ANP 10^{-3} M.

2.6. Autoradiography

Control and polycystic ovaries were removed and immediately frozen in isopentane chilled over with dry ice (−18 °C) and stored at −80 °C. The ovaries were mounted on cryostat chucks, and 16-μM sections were prepared at −16 °C. Autoradiography